Int J Clin Exp Med 2014;7(12):5252-5259
www.ijcem.com /ISSN:1940-5901/IJCEM0003495

Original Article

Intervention of transforming pulmonary fibrosis with NF-κB p65 antisense oligonucleotide

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Received October 31, 2014; Accepted November 13, 2014; Epub December 15, 2014; Published December 30, 2014

Abstract: Objective: NF-κB, especially p65 subunit, plays important role in the process of pulmonary fibrosis. In this study, we transformed fibroblast into myofibroblast induced by bleomycin, and then studied the effects of NF-κB p65 antisense oligonucleotide on pulmonary fibrosis in mouse model. Methods: Pulmonary fibrosis was induced by bleomycin in C57BL/6 mouse (modeling group). The NF-κB antisense oligonucleotide was injected intravenously into mouse 6 hours before inducing (test group), we performed broncho-alveolar lavage and blood collecting through cardiac puncture. Bronchoalveolar Lavage Fluid (BALF) and serum from normal C57BL/6 mouse (control group) were collected for comparison. Immunohistochemistry staining of the NF-κB and α-SMA on lung tissues and cultured cells were carried out in each group, respectively. Results: The expression level of NF-κB and α-SMA were both consistently higher in modeling group when compared with control group ($P < 0.05$). Meanwhile, they were reduced significantly through the intervention of NF-κB p65 antisense oligonucleotide in the test group ($P < 0.05$). More importantly, the expression of NF-κB was positively correlated with α-SMA. Conclusion: our study suggests the potential in prevention of bleomycin-induced pulmonary fibrosis with NF-κB p65 antisense oligonucleotide.

Keywords: Fibroblast, myofibroblast, NF-κB, α-SMA, antisense oligonucleotide

Introduction

Pulmonary Fibrosis (PF) is characterized by accumulation of fibroblasts, deposition of extracellular matrix, as a results, PF is accompanied with inflammation and damage caused by tissue structural destroy [1]. Recent studies showed that there is a transforming process of fibroblasts into myofibroblasts in interstitial pulmonary cells with the deposition of large amount of extracellular matrix. On the other hand, the appearance and persistence of myofibroblasts play a key role in PF [2]. Previous reports have suggested that α-smooth muscle actin (α-SMA) is the most important specific markers of myofibroblasts [3-5].

NF-κB (nuclear factor kappa-light-chain-enhancer of activated B) is a protein complex that controls transcription of DNA. NF-κB is found in almost all animal cell types, and NF-κB is involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, oxidized LDL, and bacterial or viral antigens [6-10]. As a member of family of transcription factors, NF-κB contains five subunits: Rel (cRel), p65 (RelA, NF-κB3), RelB, p50 (NF-κB1), and p52 (NF-κB2). Among them, p65, as an important regulatory subunit, has become a potential target in the pathogenesis of pulmonary interstitial fibrosis [11]. p65 contains two potent transactivation domains within its C-terminus and is might be the strongest activator in NF-κB subunits [12].

Recently, compelling studies have indicated that NF-κB p65 is highly involved in fibrosis of organ such as liver [13-15]. All these information suggests that NF-κB acts as a central link between organ injury and fibrosis, and that it may represent a target for the prevention or treatment of fibrosis. Moreover, our previous studies indicated that NF-κB was involved in the synthesis of certain cytokines in the lung fibroblasts, and subsequently regulate myofibroblasts transforming process. To explore the pathological process of PF, we studied the effects of NF-κB on the phenotype transforming

process of fibroblasts to myofibroblasts, as well as the potential inhibition effect of transforming p65 antisense oligonucleotide. We constructed mouse model with bleomycin-induced pulmonary fibrosis and injected with p65 antisense oligonucleotides to explore our study.

Materials and methods

Material and regents

The fibrosis inducing agent bleomycin (BLM) A2 was purchased from Nippon Kayaku Co., Ltd. (batch number: Y60520). RPMI-1640 medium used for cell culture was purchased from HyClone Beijing Biochemical Products Co., Ltd. Fetal bovine serum; rabbit anti-mouse α-SMA and p65 monoclonal IgG antibody were purchased from Gibco (NY, USA), Santa Cruz Biotechnology, Inc. (CA, USA), separately. The immunohistochemistry kit was purchased from Beijing Zhongshan Golden Bridge biotechnology Co., Ltd. (Beijing, CHN). For the mouse model construction, we purchased C57BL/6 female mice of SPF level from Beijing Vital River Laboratory Animal Technology Company with weighing between 18-20 g.

Animals

The mice were randomly divided into 3 groups with 60 mice for each: (1) Modeling group, mice were treated with bleomycin A2 (5 mg/kg, dissolved in 20 μl sterile saline) through intratracheal injection; (2) Control group, mice were treated with 20 μl saline in the manner of intratracheal injection; (3) Test group, mice were intravenously injected NF-κB antisense oligonucleotides (900 μg dissolved in 200 μl saline) 6 hours before modeling. For consistency, both modeling and control groups were also intravenously injected of saline 20 μl. Mice were sacrificed seven days after modeling. All animal experiments were performed under approved protocols of the institutional animal use and care committee of China Medical University.

Preparation of lung tissues and cells

After been sacrificed by cervical dislocation, lungs of mice in 3 groups were resected and immersed in formalin to fix, embedded with paraffin, and then sections were prepared for further observation. Immunohistochemistry staining against P65 and α-SMA were performed. Bronchial lavage and cardiac puncture blood collection were performed on 10 mice from each group and under strict sterile conditions; BALF and serum were collected for further analysis.

On the other hand, 5 normal mice were sacrificed by cervical dislocation. Lung tissue of these mice were taken under sterile conditions and cut into the blocks with approximately 2 mm × 2 mm. After washing 3 times with PBS, they were evenly placed on the bottom of the flask under 37°C, with 5% carbon dioxide. They were culture with RPMI-1640 medium containing 20% fetal bovine serum, 100 U/mL of penicillin and 100 U/mL of streptomycin, the medium were renewed every 2-3 days.

After cells formed in monolayer surrounding lung tissue in 1-2 weeks, they were digested with 0.25% trypsin and separated into two flasks. The 4th generation of cells was transferred into a 24-well plate. The BALF and serum collected from different groups in the previous step were added to the culture supernatant until the cells covered the whole coverslips.

Immunohistochemistry analysis

The monolayer cells were fixed with 4% paraformaldehyde, and cultured with antibody against p65 and α-SMA (1:100 dilute) according to the user guide of immunohistochemistry kit, respectively. PBS was used instead of primary antibody as negative control. The coloring was carried out with diaminobenzidine (DAB).

Microscopic image analysis system (MetaMorph/Evolution Mp5.0/BX51; UIC/OLYMPUS, US/JP) were applied for photographing. 5 pictures were taken on each slide from cultured cells under the microscope (× 400), while the amount of p65 and d α-SMA were calculated as the average value optical density (MOD).

Statistical analysis

Statistical analysis was performed with SPSS-13.0 software; all data are represented as mean ± SD. Differences between groups were compared by using the t test and the chi-square test. Linear regression models were used to evaluate the associations between expression of p65 and d α-SMA. A significant difference was considered for $P < 0.05$.

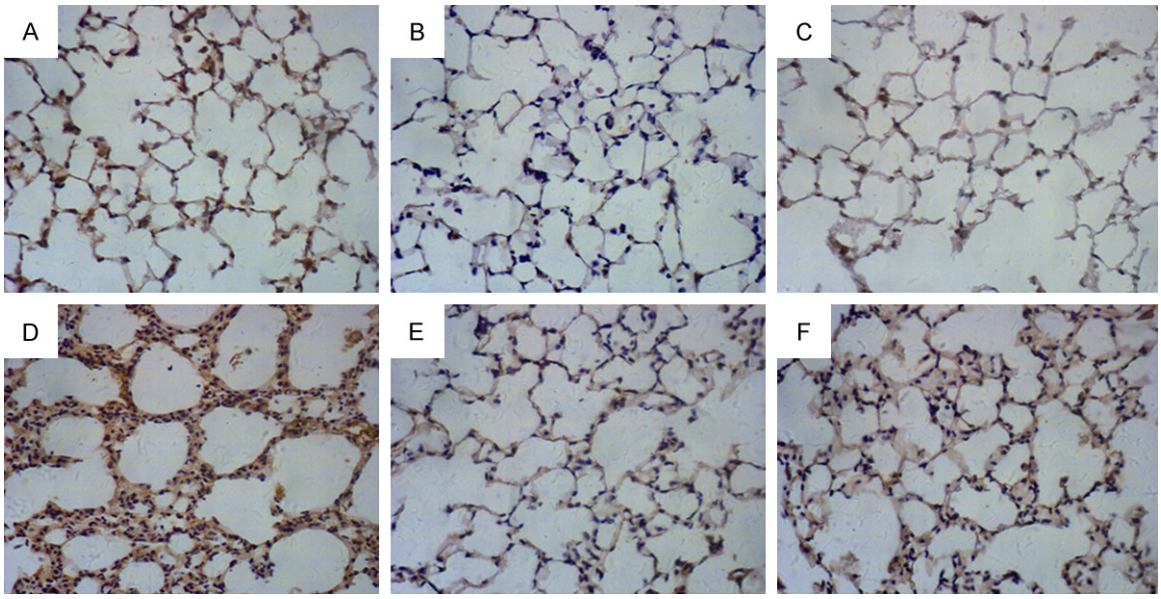

Figure 1. The immunohistochemistry staining results of P65 and α-SMA in lung tissue (× 400). A. P65 in modeling group, more brown granules could be seen in the cytoplasm and more nuclear were stained; B. P65 in control group, almost no brown granules in cytoplasm. C. P65 in test group, positive staining decreased compared with model group but intensified as compared with control group; D. α-SMA in modeling group, positive deep brown staining could be seen in the cytoplasm; E. α-SMA in control group, fewer positive staining when compared with modeling group; F. α-SMA in test group, positive staining decreased compared with model group but intensified as compared with control group.

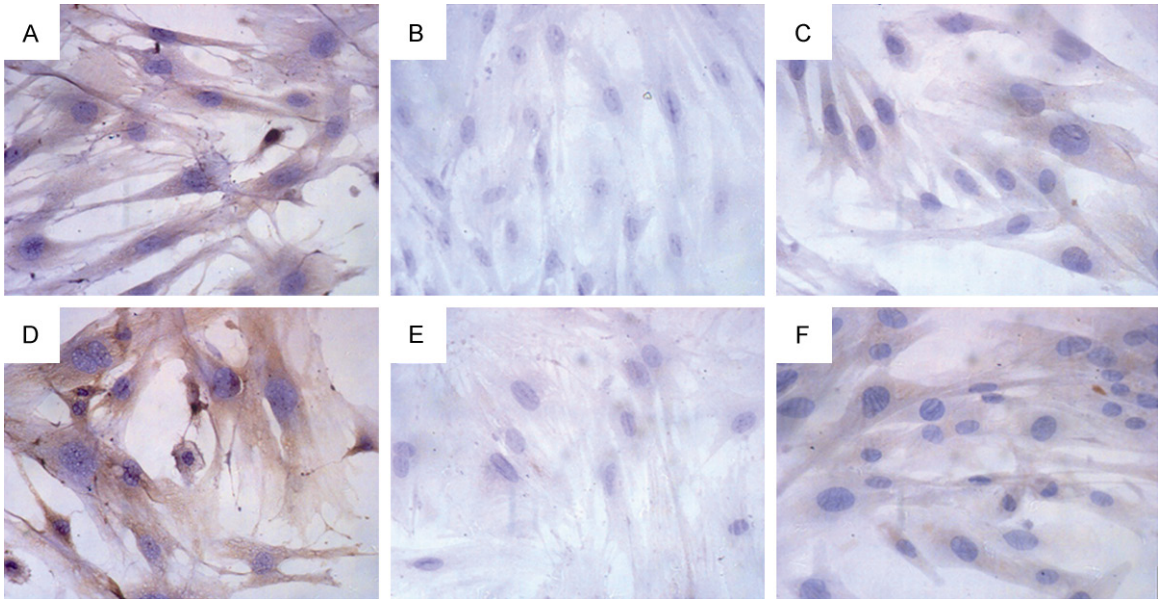

Figure 2. The immunohistochemistry staining results of P65 in primary lung fibroblast cells (× 400). A. Treated with serum in model group, deep positive stain could be seen in cytoplasm and partial nuclear; B. Treated with serum in control group, almost no staining could be seen in cytoplasm and nuclear; C. Treated with serum in test group, positive staining decreased compared with model group but intensified as compared with control group; D. Treated with BALF in model group, deep positive stain could be seen in cytoplasm and partial nuclear; E. Treated with BALF in control group, almost no staining could be seen in cytoplasm and nuclear; F. Treated with BALF in test group, positive staining decreased compared with model group but intensified as compared with control group.

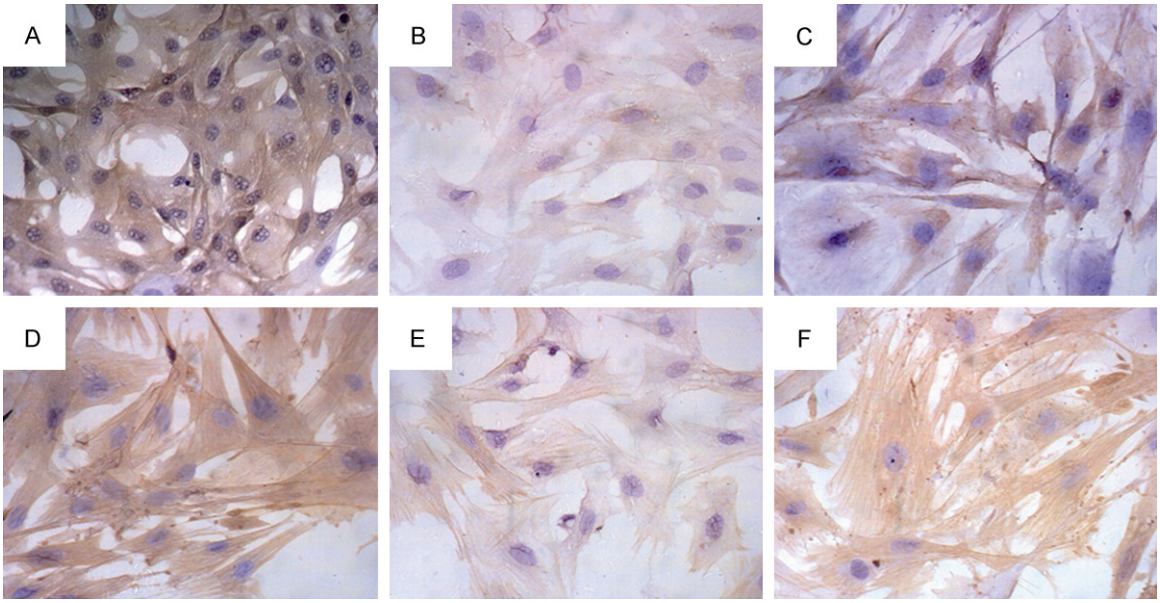

Figure 3. The immunohistochemistry staining results of α-SMA in primary lung fibroblast cells (× 400). A. Treated with serum in modeling group, deep positive brown stain could be seen in cytoplasm; B. Treated with serum in control group, positive staining could be seen in cytoplasm but decreased when compared with modeling group; C. Treated with serum in test group, positive staining decreased compared with model group but intensified when compared with control group; D. Treated with BALF in model group, deep positive stain could be seen in cytoplasm; E. Treated with BALF in control group, positive staining could be seen in cytoplasm but decreased when compared with modeling group; F. Treated with BALF in test group, positive staining decreased compared with model group but intensified when compared with control group.

Results

Comparison of p65 expression in lung tissues

The p65 staining results of lung tissue were shown in **Figure 1A-C**. As we can see, the dark brown staining could be observed extensively in the cytoplasm and nuclei of the modeling group (**Figure 1A**), which suggests NF-κB p65 expression in lung tissue was significantly enhanced after intratracheal instillation of BLM. No brown staining could be observed in cytoplasm of the control group (**Figure 1B**), which indicates that NF-κB expressed little in the normal mouse lung tissue. The staining intensity of p65 in the test group was significantly weakened when compared with modeling group while still stronger than control group (**Figure 1C**), which suggests intravenous injection of p65 antisense oligonucleotides could largely, although not completely, inhibit the expression of NF-κB induced by BLM in lung tissue.

Comparison of α-SMA expression in lung tissues

The α-SMA staining results for lung tissue were demonstrated in **Figure 1D-F**. For control group, α-SMA was considerably expressed in the cytoplasm according to the evenly distributed light brown stains (**Figure 1E**). The staining cells in the modeling group were significantly increased and the intensity was largely enhanced compared with control group (**Figure 1D**). These results suggest the basal level of α-SMA expression in lung cells of normal mouse was enhanced by intratracheal instillation of BLM. The staining intensity of α-SMA in the test group was significantly weakened when compared with modeling group but still stronger than control group (**Figure 1F**), which suggest intravenous injection of p65 antisense oligonucleotides could largely inhibit the expression of α-SMA induced by BLM in lung tissue.

Comparison of p65 expression in primary lung fibroblasts

The p65 staining results in primary lung fibroblasts were shown in **Figure 2F**. For cells treated with both serum and BALF from control group, there almost no stains could be found in both cytoplasm and nuclear (**Figure 2B-E**). As a comparison, when added serum and BALF from modeling group, more dark stains could be observed both in cytoplasm and nuclear (**Figure**

Table 1. The average optical density values (Mean ± SD) of immunohistochemistry staining of lung tissues in 3 groups

Group	N	p65 protein	α-SMA Protein
Modeling	40	0.1422 ± 0.0325[a]	0.1259 ± 0.0198[a]
Test	40	0.0988 ± 0.0241[a,b]	0.0846 ± 0.0201[a,b]
Control	40	0.0524 ± 0.0201	0.0598 ± 0.0152
F value		146.385	284.689
P value		< 0.05	< 0.05

[a]P < 0.05, compared with control group; [b]P < 0.05, compared with modeling group.

2A, **2D**). However, when we further added serum and BALF from test group which was complemented with p65 antisense oligonucleotides, results showed the staining intensity was significantly reduced as compared with modeling group but was little stronger than that of control group (**Figure 2C**, **2F**).

Comparison of α-SMA expression in primary lung fibroblasts

The α-SMA staining results of primary lung fibroblasts were shown in **Figure 3A-F**. For cells treated with both serum and BALF from control group, we observed almost no stains in either cytoplasm or nuclear (**Figure 3B**, **3E**). As a comparison, when added serum and BALF from modeling group, more dark stains could be observed in both cytoplasm and nuclear (**Figure 3A**, **3D**). However, when we further added serum and BALF from test group, the staining intensity was significantly reduced as compared with modeling group but was a little stronger than that of control group (**Figure 3C**, **3F**).

Correlation of the p65 and α-SMA expression

The mathematical statistic results were shown in **Tables 1** and **2** for both modeling and test groups. The average values in each group were compared to quantify their relationship. Results showed that there's a positive correlation of protein expression between p65 and α-SMA in the lung tissue ($r = 0.964$, $P < 0.05$). It's also shown a positive correlation of the protein expression between p65 and α-SMA in the primary lung fibroblasts treated with different interventions ($r = 0.948$, $P < 0.05$).

Discussion

Pulmonary fibrosis is a disease with extremely complex etiology and aggressive progression with poor prognosis; so far, there is no effective treatment available in the clinic, PF has become a serious threat to the human health. The transforming of fibroblasts to myofibroblasts plays very important role in the pathogenesis of pulmonary fibrosis. However, it remains unclear how transforming process is regulated.

Lung fibroblasts synthesize and secrete a variety of cytokines, which including collagen and matrix proteins. In this way, fibroblasts could form a positive feedback with self-excitation in response to inflammation, and thereby enhance the effect of duration and aggravation of fibrosis [16]. The synthesis and secreting ability of fibroblasts could increase significantly when they transformed into myofibroblasts. Meanwhile, myofibroblasts with contractile properties could cause changes in the mechanical properties of fibrotic lung tissue, which leads to the reduction of lung compliance. Studies have suggested these characteristics are related with the increased expression of α-SMA [3, 17].

The p65, as a subunit of the essential transcription factor NF-κB, contributes much to the main function of NF-κB. NF-κB exists in the cytoplasm at the inactive form and become active when enters into the nucleus, through binding to the promoter region of target gene to initiate the transcription. Few literatures repot the function of NF-κB in regulating synthesis of α-SMA. However, numerous studies indicated there's a close relationship between them. For example, TNF-α, TGF-β, IL-1β and PDGF were all involve in the activation, chemotaxis and mitogenesis process, as well as stimulating the synthesis of collagen in lung fibroblasts [18, 19]. Upon activation, NF-κB involved in the transcriptional regulation of numerous cytokine genes and inflammatory reaction, as a result, which lead to stimulating production of lung fibroblasts [20, 21].

According to our previous studies, the expression of NF-κB is highly relates with the activation and transformation of fibroblasts, which leads to pulmonary fibrosis. To prevent the transforming of BLM-induced interstitial fibrosis in lung with the NF-κB antisense oligonucleotide, we combined immunohistochemistry and immunocytochemistry techniques to compare the expression of NF-κB in different mouse models. We collected BALF and serum from both groups and added them into primary lung

Table 2. The average optical density values (Mean ± SD) of immunohistochemistry staining in primary lung fibroblasts of 3 groups

Origin of intervention regents	N	p65 Protein		α-SMA Protein	
		Serum	BALF	Serum	BALF
Modeling group	40	0.1358 ± 0.0256[a]	0.1426 ± 0.0442[a]	0.1299 ± 0.0407[a]	0.1368 ± 0.0164[a]
Test group	40	0.0965 ± 0.0241[a,b]	0.1054 ± 0.0348[a,b]	0.0883 ± 0.0231[a,b]	0.0750 ± 0.0098[a,b]
Control group	40	0.0386 ± 0.0201	0.0397 ± 0.0256	0.0487 ± 0.0104	0.0442 ± 0.0206
F value		296.584	389.455	455.658	541.356
P value		< 0.05	< 0.05	< 0.05	< 0.05

Abbreviation: BALF, BronchoAlveolar Lavage Fluid. [a]P < 0.05, compared with control group; [b]P < 0.05, compared with modeling group.

fibroblasts of normal mice, then compared the NF-κB expression and analyzed the correlation between NF-κB and α-SMA.

Our results showed that expression of p65 was significantly increased in mice with pulmonary fibrosis compared to normal mice, and the expression of α-SMA was up-regulated than normal mice, which is consistent with previous studies [22, 23]. p65 antisense oligonucleotides could inhibit the expression of p65 and α-SMA induced by BLM. The expression of p65 and α-SMA showed a significant high correlation ($r = 0.964$, p-value < 0.05), this further confirms our hypothesis that NF-κB functions as regulator for transforming of fibroblasts into myofibroblasts during the pathology of BLM-induced lung injury, which further leads to pulmonary fibrosis. Furthermore, the antisense oligonucleotide of NF-κB could play an important role in preventing pulmonary interstitial fibrosis pathogenesis.

RNAi technology is based on the principle of complementary hybridization of nucleic acid to specifically inhibit the expression of selected genes (mRNA). Antisense nucleic acid of targeted gene could bind to the specific sequences; thereby inhibit the interactions with nucleic acid, proteins, or other factors, which ultimately affect the metabolism and function of targeted gene in cells.

In the present study, we also observed studied the effects of NF-κB antisense oligonucleotide on the survival of pulmonary fibrosis induced mice. In modeling group, after two weeks' observation, the mortality rate was 20% (6/30). However, when we adopted intravenous injection of NF-κB antisense oligonucleotides 6 hours before intervention, it's surprisingly found that no mice died in the test group, It's obviously that antisense oligonucleotide improve the survival significantly. In addition, we observed the endocytosis of fluorescelabeled NF-κB antisense oligonucleotides in lung fibroblasts with fluorescence microscope. According to immunocytochemistry, we further confirmed that antisense oligonucleotide largely reduces the expression of both cellular and *in vivo* NF-κB and α-SMA, which provides basis for the inhibiting transformation of fibroblasts to myofibroblasts. These results suggest that antisense oligonucleotides could be incorporated by activated fibroblasts to reduce lung injury/fibrosis, and then improve the survival of mice bearing bleomycin-induced pulmonary fibrosis.

The *in vitro* experiments also demonstrated that the treatment of antisense oligonucleotide could reduce the expression of NF-κB and the transforming of fibroblasts into myofibroblasts in both modeling groups. According to our previous observation results (data not show), a large amount of fluorescein-labeled NF-κB antisense oligonucleotide accumulates in the peripheral blood mononuclear cells and alveolar macrophages. Meanwhile, reports have indicared that NF-κB antisense oligonucleotide can significantly inhibit the expression of TNF-α and IL-4 in BALF and serum, which are closely related with pulmonary fibrosis [24, 25]. These results highly suggested that NF-κB antisense oligonucleotides functions through reducing the expression of NF-κB as well as inhibiting the transcription of NF-κB-related inflammatory factors to prevent the activation of fibroblasts and transforming into myofibroblasts.

Presently, the primary drug in the treatment of pulmonary fibrosis is corticosteroid and/or immunosuppressive agents, whose therapeutic potential is limited with considerable side effects. Exploring more effective and efficient

drugs is very necessary for pulmonary fibrosis treatment. We initially proved that NF-κB antisense oligonucleotides prevent the transforming of fibroblasts into myofibroblasts effectively, and have satisfied therapeutic effect in the treatment of pulmonary fibrosis *in vivo*, this provide some useful reference for the treatment of pulmonary fibrosis in the future.

Acknowledgements

The authors thanks the support work given by our colleague.

Disclosure of conflict of interest

None.

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