C/EBPβ Acetylation is Involved in Idiopathic Pulmonary Fibrosis

Jingzhu Zhou
yixing people's hospital
Xiuhai Ji
taicang hospital of tranditional chinese medicine
yan fen
yixing people's hospital
Hui ding (dh1350519@163.com)
yixing people hospital affiliated jiangsu university https://orcid.org/0000-0003-2477-461X

Research

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Abstract

Background: IPF is a progressive lung disease, characterized by excessive deposition of ECM. C/EBPβ is involved in the development of pulmonary fibrosis. However, the regulation of C/EBPβ in the context of pulmonary fibrosis is not clear. The study is to identify the C/EBPβ acetylation in IPF

Methods: Lung from six IPF and six control samples were selected in this study. We investigated the expression of C/EBPβ in lungs with Immunochemistry. Moreover, the expression of C/EBPβ mRNA via Real Time-PCR and its protein expression via Western Blot were performed. Meanwhile, the levels of collagen-I and α-SMA as markers of pulmonary fibrosis were also determined by Western Blot. Furthermore, we confirmed the relationship between α-SMA and acetylated C/EBPβ by Co-Immunoprecipitation.

Results: We found the elevated C/EBPβ mostly locating in fibroblast foci in lungs of IPF. And the expression of C/EBPβ RNA and protein were obviously increased in IPF ($P < 0.05$), in which the proteins of α-SMA and collagen-I were enhanced ($P < 0.05$). Furthermore, the stronger acetylation of C/EBPβ binging to the α-SMA gene was shown in lung fibrosis ($P < 0.05$).

Conclusions: The increased expression of C/EBPβ acetylation associated with α-SMA expression is involved in the development of pulmonary fibrosis.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic fibrotic lung disease characterized by usual interstitial pneumonia (UIP) (1). The prognosis of IPF with 2–5 years survival lifetime after diagnosis is remains poor. However, the exact mechanism is unclear. It was accepted that the abnormal recovery of injured alveolar epithelial cells participated in the process of pulmonary fibrosis (2). Recent study suggested that the myofibroblasts from alveolar epithelial-mesenchymal transition (EMT) produced aberrant cytokines and excessive deposition of extracellular matrix (ECM), following with the subsequent distortion of lung homoeostasis and architecture (3,4). The expression of α-smooth muscle actin (α-SMA) as a marker of mesenchymal cells were increased in A549 cell with profibrotic factors, resulting into Collagen-I over-generation as fibrotic models in vitro (5).

As an important transcription factor, CCAAT/enhancer binding protein β (C/EBPβ) plays roles in cell differentiation and cytokine secretion (6). The previous study demonstrated that C/EBPβ expression was significantly increased in bleomycin-induced pulmonary fibrosis (7). In addition, C/EBPβ knockout mice antagonized the fibrotic roles of bleomycin in lungs as myofibroblast aggregation and collagen deposition (8). Indeed, phosphorylation of C/EBPβ was reported to be as an important step in its regulation effect on pulmonary fibrosis (9,10). However, the acetylation of C/EBPβ in adjusting epithelial cell function and transition involved in pulmonary fibrosis is unknown.

In this paper, we aimed to identify the expression of C/EBPβ and its acetylation in lung tissue of IPF patients. Furthermore, the relationship between C/EBPβ acetylation and α-SMA gene expression was investigated.

Material And Methods

Human lung samples

All lung tissues were obtained from surgical resections at Yixing people hospital affiliated Jiangsu University. This study was approved from the institutional review boards (IRBs) at the Yixing People's Hospital. The informed consent obtained from all subjects was confirmed. IPF was diagnosed in accordance with the criteria of the American Thoracic Society and European Respiratory Society (1). These IPF patients included 3 female patients and 3 male patients, and the average age was 52±11 years. Control samples were from 2 female and 4 male patients with average age 47±18 years. All tissue samples were immediately frozen in liquid nitrogen and kept at -80 °C.

Quantitative real-time RT-PCR

Total RNA from frozen tissues was isolated using Trizol reagent (Life Technologies, USA) according to the manufacturer's instruction. Quantitative real-time RT-PCR was performed using SYBR Green (Takara, China) on a Real-Time Quantitative Thermal Block (Biometra, Germany). The integrity of 300ng RNA was used to each assay. Primer sequences used for C/EBPβ were forward, 5'-GCCTCTCCACGTCCTCCTCGT-3', and reverse, 5'-CGCTGTGCTTGTCCACGGTCT-3'. Primer sequences used for the internal control of GAPDH were forward, 5'-CACCTTCACCGTTCCAGTTT-3' and reverse, 5'-CTCTTCCAGCCTTCCTTCCT-3'. The reactions were carried out at 95 °C for 30 s, then 94°C for 45 s, 60°C for 45 s, and 35 cycles of 72 °C for 5 min, and a final extension at 72 °C for 10 min. Determination of C/EBPβ mRNA was normalized to GAPDH as controls.

Immunohistochemistry

Four percent paraformaldehyde-fixed, paraffin-embedded blocks of lung tissues were cut into 4-μm sections. After deparaffinating, rehydration and retrieval, tissues were incubated with an antibody against C/EBPβ (Santa Cruz, USA) overnight at 4°C. After being washed by PBST 3 times with each 15 minutes, the sections were incubated with the corresponding second antibody at room temperature for 1 hour. The results were visualized with diaminobenzidine. Positive signals showing brown were diagnosed by glycerin mount preparations.

Western Blot

After being separated on 10% SDS-PAGE gels, the proteins were transferred to PVDF membranes and then blocked with 5% nonfat milk in TBST. The time of incubating with primary antibody is overnight at 4°C. Primary antibodies against α-SMA and Collagen I were purchased from Abcam. Antibodies against C/EBPβ were purchased from Santa Cruz. Membranes were incubated with appropriate secondary

antibodies. The chemiluminescence signal captured on X-ray film and quantified by Quantity One software was developed by ECL detection kit.

Co-Immunoprecipitation

Protein samples were immunoprecipitated with either polyclonal antibody against C/EBPβ (Santa Cruz, USA) or control IgG (Santa Cruz, USA) at 4°C overnight. Then samples were constantly agitated with A/G-agarose beads (Santa Cruz, USA) at 4°C for 4h. After being washed 5 times with buffer, beads were prepared to exact proteins. Proteins from deposits with lysate were used for Western Blot as above. Antibodies used in the experiments were Acetylated-Lysine Antibody (Santa Cruz, USA and α-SMA (Abcam, United Kingdom).

Statistical analysis

These data were presented as the Mean ± SEM. Differences between groups were calculated by one-way ANOVA analysis with SPSS software. A value of $P < 0.05$ was considered statistically significant.

Results

Elevated expression of C/EBPβ mRNA in IPF

In order to understand the roles of C/EBPβ in pulmonary fibrosis, it's firstly necessary to confirm the expression of C/EBPβ gene between IPF and controls. In this study, C/EBPβ mRNA expression was elevated in IPF by approximately 2-fold compared with controls (Fig.1). So, the difference of C/EBPβ mRNA expression maybe associated with the fibrotic process of pulmonary.

Increased C/EBPβ expression locating at fibroblasts foci in IPF

C/EBPβ phosphorylation has been reported to play critical roles in alveolar EMT and pulmonary fibrosis (9). Therefore; we sought to determine whether C/EBPβ expression is increased in lung tissues of IPF. And it's more important to exhibit the position of C/EBPβ in order to clearly understand the function on the onset and development of pulmonary fibrosis. As shown in Fig.2, C/EBPβ expression marked brown color as positive reaction was significantly increased. The remarkable performance of pulmonary fibrosis is the deposition of fibroblast foci consisted with myofibroblasts, the major player in secreting ECM. We found that the presence of C/EBPβ mostly gathered near fibroblast foci. Nevertheless, the increased C/EBPβ potentially participated in the ECM deposition and pulmonary fibrosis.

Enhanced expression of C/EBPβ protein and its acetylation in IPF

The topical pathological feature of IPF is ECM deposition, such as fibronectin and collagen-I, which causes abnormality of lung tissue resulting into pulmonary function insufficiency. Furthermore, intensive expression of α-SMA as a marker of myobroblast was present in fibrotic lung tissues. Indeed, the collagen-I and α-SMA were obviously elevated in lung tissues of IPF. In this study, we confirmed that the

expression of C/EBPβ mRNA was raised rapidly. Moreover, the expression of C/EBPβ was also increased at protein levels (Fig. 3).

Although the change of C/EBPβ expression in IPF were confirmed, it's not sufficient to explain the clear function in the advancing of disease. The previous research reported the phosphorylated C/EBPβ was involved in the EMT and fibrosis [9]. In our study, we firstly revealed that acetylated C/EBPβ was significantly enlarged in lung tissues of IPF, as shown in Fig.4. Taken together, these results indicated that augmented C/EBPβ might play roles in the development and progress of pulmonary fibrosis via acetylated modification.

Acetylated C/EBPβ binding to α-SMA in IPF

The previous study demonstrated an essential effect in pulmonary fibrotic stage that mice lacking C/EBPβ showed an antagonism with bleomycin-caused lung fibrosis [8]. We hypothesized that acetylation of C/EBPβ-induced binding to α-SMA gene activity efficacy is involved in the deposition of ECM and development of lung fibrosis. We further verified the association between acetylated C/EBPβ and α-SMA by Co-Immunoprecipitation. As shown in Fig.4, the increased α-SMA expression was revealed in samples with C/EBPβ antibody detected by acetylated antibody, even not in IPF tissues with IgG antibody. Hence, it's proved that the acetylated modification of C/EBPβ activated its liable α-SMA expression in IPF.

Discussion

IPF is a worldwide severe disease with limited choice for treatments and poor prognosis. At present, it was accepted that myofibroblasts mostly from abnormal alveolar epithelial cells repair as EMT was responsible for the deposition of extracellular matrix and destruction of the lung architecture (2). As the eminent effecter producing Collagen fibers, myofibroblasts marked with α-SMA constituted fibroblast foci, which was the topical histological performance in IPF (11). Our present study not only showed the change of histological architecture in IPF via Immunohistochemistry, but also confirmed the increased expression of α-SMA and collagen-I in lung tissues via Western Blot. Hence, the investigation into molecular mechanism of α-SMA and collagen-I regulation in pulmonary fibrosis is especially important. In this study, we firstly disclosed that stronger acetylation of C/EBPβ was responsible for α-SMA expression in lung tissues of IPF.

With the regulation roles of cellular grow, differentiation and pro-inflammatory cytokine secretion, C/EBPβ is present in organs, as lung, liver, kidney and so on (12). The intense research of C/EBPβ function in lung is desirable. Indeed, Yan et al (13) found that C/EBPβ was involved in the regulation of lung epithelial cell differentiation and proliferation for the protection during acute lung injury. Miglino's study revealed that cigarette smoke-induced increased C/EBPβ expression was critical to the pro-inflammatory factor activation (14). Indeed, the role of C/EBPβ in maintaining the stability of epithelial cells is observable. Hu et al (8) reported activation of C/EBPβ was essential for the secretion of cytokines and the differentiation of myofibroblasts in vivo lung. The participation of C/EBPβ in lung cells suggested its potential roles in

pulmonary fibrosis. The previous study showed that increased C/EBPβ expression was appeared in pulmonary fibrotic model in mice with bleomycin (15). However, there's no direct report in human lung tissues. Hence, we firstly confirmed the elevated C/EBPβ expression in lung tissues of IPF. In histological exhibition, enhanced C/EBPβ mainly located at areas of fibroblast foci. Furthermore, the expression of C/EBPβ mRNA and protein in IPF were significantly increased, suggesting that C/EBPβ might be closely related to the development of pulmonary fibrosis. As reported, C/EBPβ played key roles in EMT and pulmonary fibrosis in vivo and in vitro (7,15). As the recognized pro-fibrotic factor, the level of transformed growth factor-beta (TGF-β) was decreased in bleomycin-treated C/EBPβ deficient mice (7), failed into α-SMA and collagen-I sufficient expression. The data suggested that an essential effect in pulmonary fibrotic stage that mice lacking C/EBPβ showed an antagonism with bleomycin-caused pulmonary fibrosis (8). The effect of C/EBPβ gave us a clue to further investigate its biological mechanism in pulmonary fibrosis.

As an important transcription factor, the function of C/EBPβ is activated by various modifications, such as phosphorylation, acetylation and mythylation. Lin et al reported that C/EBPβ phosphorylation played core roles in thrombin-induced IL-8 release in lung epithelial cells (16). And Martina et al (9) demonstrated that inhibition of C/EBPβ phosphorylation blocked the progression of bleomycin-induced lung injury and fibrosis. Schwartz et al (17) confirmed the acetylation of C/EBPβ is necessary for the cytokine activation and its-mediated transcriptional function. To our knowledge, the relationship between C/EBPβ acetylation and pulmonary fibrosis has not been reported. Our study found for the first time that acetylated C/EBPβ expression was significantly enhanced in lung tissues of IPF.

There's reported that acetyltransferase p300 successfully acetylated C/EBPβ at Lysine 39 (17). Indeed, p300 was required for enhanced α-SMA expression in fibroblast biology and fibrosis (18). C/EBPβ was confirmed to have a role on α-SMA promoter via binding interactions in Interleukin-β (IL-β) secretion (19). We hypothesized that acetylation of C/EBPβ-induced binding with α-SMA gene activity in IPF. In our study, we firstly and directly showed that acetylation of C/EBPβ taking a binding with α-SMA expression in pulmonary fibrosis. The acetylation of C/EBPβ may regulate the expression of α-SMA, resulting in the deposition of ECM. And the exact mechanism of C/EBPβ provoking its activation liable for the α-SMA expression needs further study.

Conclusion

We firstly demonstrated that the enhanced expression of C/EBPβ and its acetylation were present in lungs of IPF. Moreover, acetylated C/EBPβ appeared to be responsible for α-SMA gene activation. The C/EBPβ acetylation may be critical for the progression of pulmonary fibrosis.

Abbreviations

IPF: Idiopathic pulmonary fibrosis; ECM: extracellular matrix; C/EBPβ: CCAAT/enhancer binding protein β; α-SMA: α-smooth muscle actin; UIP: usual interstitial pneunomia; EMT: epithelial-mesenchymal transition;

TGF-β: transformed growth factor-beta; IL-β: Interleukin-β.

Declarations

Ethics approval and consent to participate

This study was approved from the institutional review boards (IRBs) at the Yixing People's Hospital.

Consent for publication

All author approved to publish the paper. All presentations of patients have consent to publish.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Jingzhu Zhou and Hui Ding wrote the manuscript. Jingzhu Zhou and Jixiu Hai carried out the study. Ruhua Chen and Yan Fen prepared the samples. All authors read and accepted the manuscript.

Availability of Data and Materials

The authors ensure the availability of supporting data and materials.

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Figures

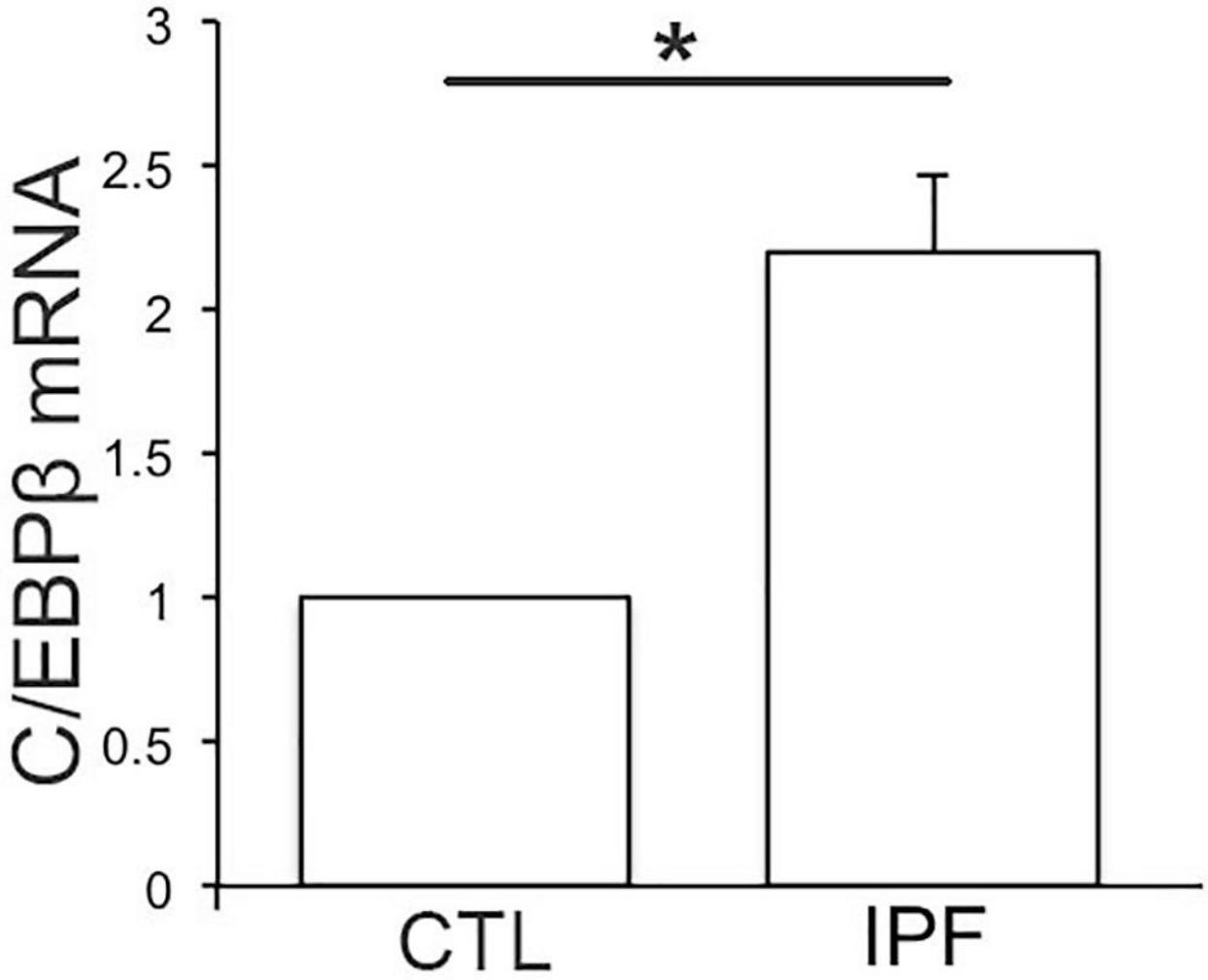

Figure 1

Levels of C/EBPβ in lung tissues of IPF. The expression of C/EBPβ mRNA was significantly increased in lung tissues of IPF compared with controls. *p < 0.05

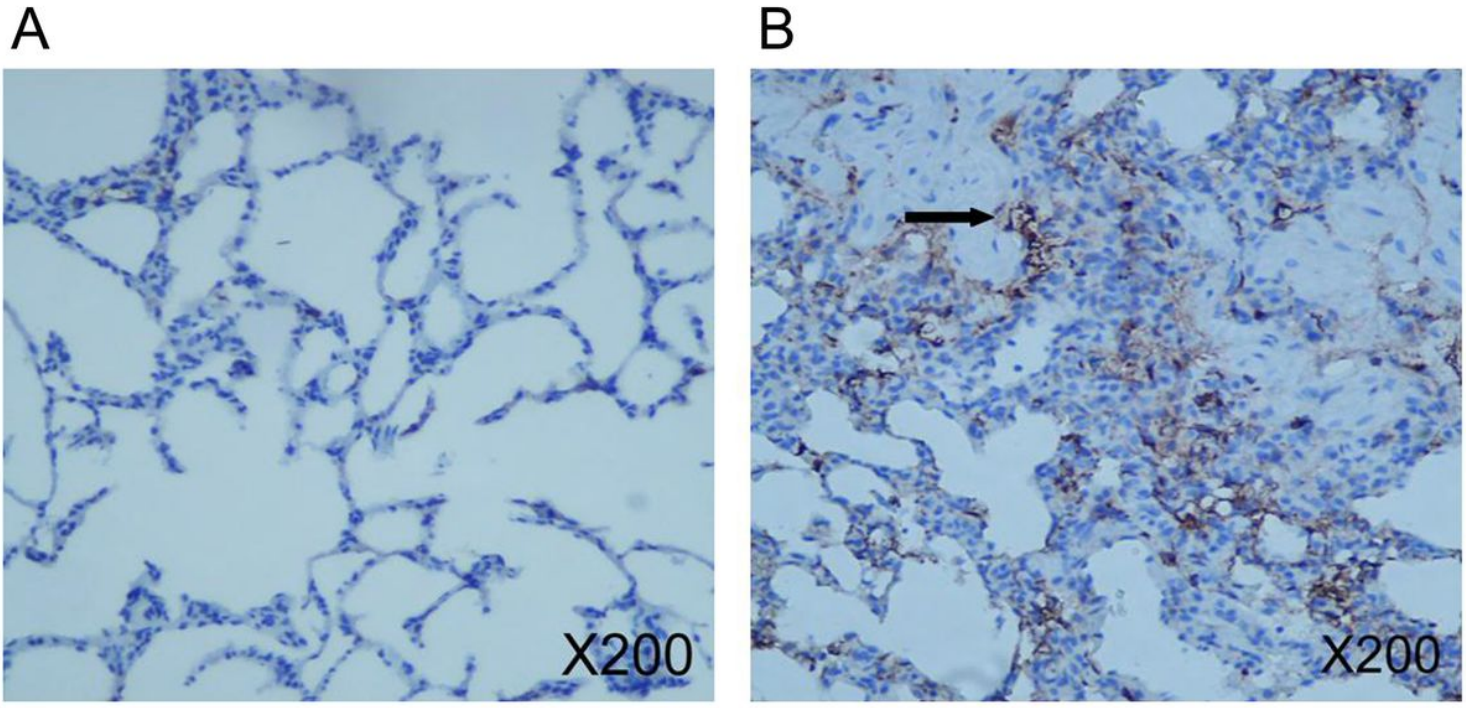

Figure 2

Representative immunohistochemical staining for C/EBPβ expression in IPF. (A) Immunohistochemical Staining for C/EBPβ expression in normal lung, original magnification, ×200; (B) Immunohistochemical Staining for C/EBPβ expression mostly locating in fibroblast foci in lungs of IPF, shown as black arrow, original magnification, ×200.

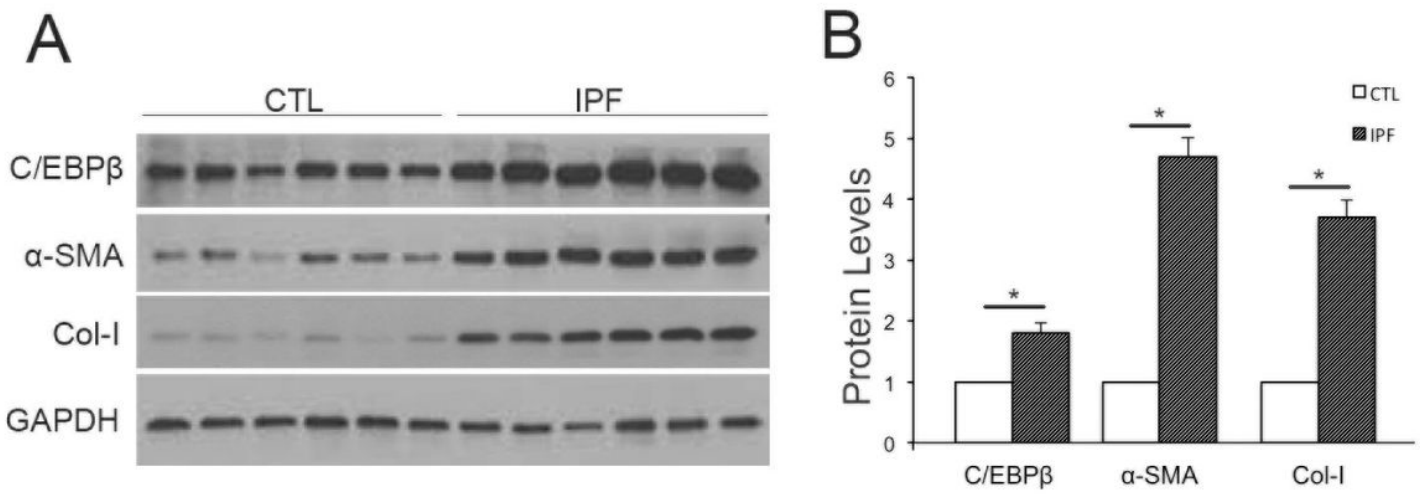

Figure 3

Elevated expression of C/EBPβ protein was present in IPF. (A) As indictors of fibrosis, α-SMA and Col-I protein expressions were accelerated in lung tissues of IPF (n=6). C/EBPβ protein revealed an obvious growing in IPF. *p < 0.05 (B) Densitometry with band intensities of C/EBPβ protein was increased in IPF by nearly 2-fold compared with controls.

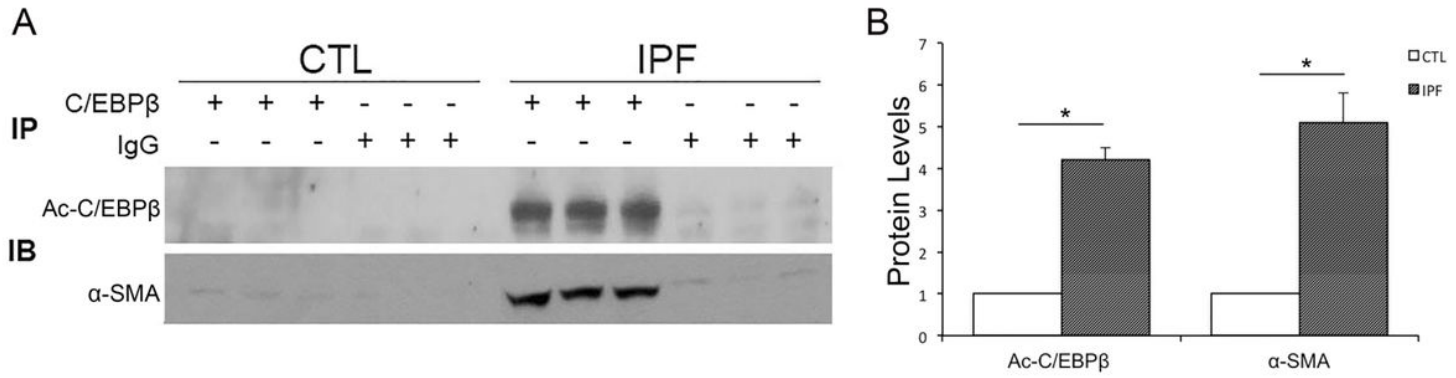

Figure 4

Acetylated C/EBPβ binding to α-SMA in IPF. (A) The acetylation C/EBPβ was significantly enlarged in lung tissues of IPF. The expression of α-SMA was bound to C/EBPβ acetylation. *P < 0.05. (B) The acetylation of C/EBPβ in IPF was nearly 4-fold compared with controls.