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ScienceDirect
Experimental and Molecular Pathology 83 (2007) 367–376
Experimental and Molecular Pathology
www.elsevier.com/locate/yexmp

# p63 – Key molecule in the early phase of epithelial abnormality in idiopathic pulmonary fibrosis

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Received 4 January 2007, and in revised form 19 March 2007
Available online 10 April 2007

**Abstract**

Idiopathic pulmonary fibrosis (IPF) is the most common lung disease predisposing lung cancer. To clarify the early phase of epithelial abnormalities in IPF, we used an *in vitro* squamous metaplasia model, transforming growth factor β1 (TGF β1)-treated airway epithelial cells (BEAS-2B). The model repeated the expression of squamous epithelial character, such as involucrin, and keratin 6 and 14. DNA microarray analysis disclosed a unique expression signature in TGF β1-treated airway epithelial cells, 20 specifically up-regulated genes including *p63*, *jagged 1* (*jag1*) and the genes of structure proteins. Western blotting and RT–PCR analysis revealed that ΔNp63α was the dominant isoform of p63 in our experimental model. Immunohistochemical analysis demonstrated the expression of p63 and jag1 in lung tissues of IPF. Inhibition of p63 with siRNA caused the down-regulation of jag1 expression, but not of involucrin, or keratin 6 and 14. Interestingly, the up-regulation of p63 was totally suppressed by *N*-acetyl-L-cysteine (NAC), but not by dexamethasone or pirfenidone. Thus, the p63–jag1 pathway may be up-regulated at an early phase of epithelial abnormalities in IPF, which can be overcome by NAC even in the TGF β1-rich milieu.


*Keywords:* Dexamethasone; Idiopathic pulmonary fibrosis; *jagged 1*; Lung carcinoma; *N*-Acetyl-L-cysteine; p63; Pirfenidone; Squamous metaplasia; Transforming growth factor β

## Introduction

Idiopathic pulmonary fibrosis (IPF) is the most common lung disease predisposing lung cancer (Hironaka and Fukayama, 1999; Hubbard et al., 2000). IPF patients have an 8- to 14-fold increased risk of lung cancer (Aubry et al., 2002; Hubbard et al., 2000; Kawasaki et al., 2001a, 2002; Park et al., 2001), and 10% of IPF patients die of lung cancers (Panos et al., 1990). Usual interstitial pneumonia (UIP) is the pathology of the lung tissues of IPF patients (ATS and ERS, 2000). UIP is also described as cryptogenic fibrosing alveolitis, and the condition is characterized by progressive lung fibrosis with spatial and temporal heterogeneity, and by remodeling of the peripheral air spaces with the disordered proliferation of epithelial cells (ATS and ERS, 2000). The histological features of epithelial changes in the honeycombed area could be classified into three categories: squamous metaplasia, cuboidal cell metaplasia, and bronchial cell metaplasia (Hironaka and Fukayama, 1999; Meyer and Liebow, 1965). Morphometric and molecular studies on UIP/IPF lungs suggest that patients with squamous metaplasia of honeycomb lesions tend to develop carcinomas such as squamous cell carcinomas (SCC) and adenocarcinomas (Aubry et al., 2002; Hironaka and Fukayama, 1999). The relatively higher incidence of SCCs in peripheral lung was reported in UIP/IPF patients as compared with non-UIP/IPF patients (Aubry et al., 2002; Kawasaki et al., 2001a). The abnormality of *p53* genes and aberrant methylation of *p16 (INK4a)* have been detected in squamous metaplasia and dysplasia as an early event to progress lung cancer (Belinsky et al., 1998; Kawasaki et al., 2001b); however, early molecular events in

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0014-4800/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexmp.2007.03.006

metaplastic epithelial cells in UIP/IPF have not been clarified (Bouros et al., 2002). Here we focused on squamous metaplasia as an early phase of epithelial abnormalities in UIP/IPF patients.

In the pathogenesis of UIP/IPF, numerous profibrotic cytokines are secreted by macrophages, alveolar epithelium, fibroblasts, and/or myofiblasts (Keane et al., 2003). In particular, transforming growth factor β1 (TGF β1) is one of the most important factors for profibrotic effects, including the induction of myofibroblast phenotype, the increase of collagen synthesis, and the reduction of matrix metalloproteinase 1 synthesis (Desmouliere et al., 1993; Edwards et al., 1987; Fine and Goldstein, 1987; Overall et al., 1989). TGF β1 also contributes to the growth and differentiation of epithelial cells; TGF β1 or serum-supplemented medium induces the differentiation of normal bronchial epithelial cells to squamous cells (Lechner et al., 1982, 1984; Masui et al., 1986). Several therapeutic agents, such as *N*-acetyl-L-cysteine (NAC), corticosteroid, and pirfenidone (PFD), have been shown to exert a preventive effect on the progression of fibrosis in UIP/IPF patients (ATS and ERS, 2000; Azuma et al., 2005; Demedts et al., 2005). Considering the dual effects of growth factors on fibroblasts and epithelial cells, such as that seen in TGF β1, it is further interesting to investigate the effect of the therapeutic agents on epithelial cells in UIP/IPF.

In order to investigate the early molecular event of epithelial abnormalities in the development of lung cancer in UIP/IPF, we adopted an *in vitro* approach in the present study. Since TGF β1 has been shown to induce squamous differentiation in transformed immortalized bronchial epithelial cells (BEAS-2B) (Ke et al., 1988), we used this model to represent an early phase of epithelial abnormalities in UIP/IPF. The subsequent gene expression analysis has demonstrated a unique signature of squamous differentiation, especially the up-regulation of *p63* and *jagged 1* (*jag1*). In addition, NAC was shown to be effective in suppressing this pathway in our *in vitro* model.

## Materials and methods

### *Cell line and culture condition*

BEAS-2B cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained in Bronchial Epithelial Cell Growth Medium (BEGM; Cambrex, Rockville, MD, USA), which is recommended by the cell supplier, containing 52 μg/ml bovine pituitary extract, 0.5 μg/ml hydrocortisone, 0.5 ng/ml human epidermal growth factor, 0.5 μg/ml epinephrine, 10 μg/ml transferrin, 5 μg/ml insulin, 0.1 ng/ml retinoic acid, 6.5 ng/ml triiodothyronine, 50 μg/ml gentamycin and 50 μg/ml amphotericin-B (Ke et al., 1988; Lechner et al., 1982, 1984; Masui et al., 1986). The medium was replenished every 2–3 days, and the cells were passaged on a collagen type 1-coated dish (ASAHI TECHNO GLASS, Tokyo, Japan) in a humidified atmosphere with 5% $CO_2$ and 95% air.

### *Experimental cell culture*

The cells were grown until they were subconfluent, and were spread at 2200 cells/cm$^2$ in the experimental medium (BEGM without retinoic acid or epinephrine) as described previously (Ke et al., 1988; Lechner et al., 1982, 1984; Masui et al., 1986). Following overnight incubation, the cells were stimulated by recombinant human TGF β1 (R&D Systems, Minneapolis, MN, USA). Dexamethasone (D4902, DEX) and *N*-acetyl-L-cysteine (A9165, NAC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pirfenidone (PFD) was purchased from Tocris Bioscience (Avonmouth, UK). To examine the effect of each drug, the cells were pre-incubated with either vehicle, DEX, NAC or PFD for 60 min before TGF β1 stimulation.

### *Antibodies*

Six primary antibodies were used: anti-involucrin (mouse monoclonal, clone SY5; 1:200 dilution for immunohistochemistry (IHC) and 1:400 dilution for Western blotting; Novocastra, Newcastle, UK), anti-keratin 6 (mouse monoclonal, clone Ks6.KA12; 1:100 for IHC and 1:200 for Western blotting; Acris, Hiddenhausen, Germany), anti-keratin 14 (mouse monoclonal, clone LL002; 1:100 for IHC and 1:10 000 for Western blotting; Lab Vision, CA, USA), anti-p63 (mouse monoclonal, clone 4A4; 1:50 for IHC and 1:500 for Western blotting; Santa Cruz Biotechnology, CA, USA), anti-Jagged 1 (goat polyclonal, 1:200 for IHC and 1:2000 for Western blotting), and anti-actin (goat polyclonal, Santa Cruz Biotechnology, 1:1000 for Western blotting as an internal control). As secondary antibodies, we used anti-mouse IgG biotin conjugate (DakoCytomation, Glostrup, Denmark, 1:250 dilution for IHC), anti-mouse IgG peroxidase conjugate (Amersham Bioscience, Buckinghamshire, UK; 1:1000 dilution for Western blotting) and anti-goat IgG peroxidase conjugate (Sigma-Aldrich; 1:1000 dilution for Western blotting).

### *Immunohistochemistry*

The lung tissues for the immunohistochemical analysis were obtained from autopsy and surgically resected specimens from patients with UIP/IPF. All resected specimens were fixed in 10–20% formalin, embedded in paraffin, cut into 3-μm-thick sections, and deparaffinized through graded alcohol and xylene. For antigen retrieval, sections were heated in 10 mM citrate buffer for 10 min at 120 °C by autoclave treatment (pH 7.0 for p63 and pH 6.0 for anti-jag1), pH 6.0 for 15 min at 100 °C in a microwave oven (for anti-keratin 6 and anti-keratin 14) or digested with 0.1% trypsin for 20 min at room temperature (for anti-involucrin). Following overnight incubation with the primary antibody at 4 °C, endogenous peroxidase was blocked with 3% hydrogen peroxide in TBS for 20 min at room temperature. Subsequently, the sections were incubated with biotinylated secondary antibody for 30 min at room temperature, allowed to react for 10 min with avidin–biotin–peroxidase complex using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) and counterstained with hematoxylin. Immunohistochemical evaluation was independently assessed by two investigators (K. M. and S. O). All lung tissue on the slide was evaluated. Immunohistochemical expression was estimated as follows: +, >5% cell stained for anti-involucrin, anti-keratin 6, anti-keratin 14; +, two or more layers stained for anti-p63; +, stained for anti-jag1, respectively.

### *Western blotting analysis*

Cells were lysed in a lysis buffer consisting of 50 mM Tris–HCl (pH 6.8) and 2% sodium dodecyl sulfate with a cocktail of proteinase inhibitors. After passing several times through a 21-gauge needle with a syringe, lysates were boiled at 95 °C for 5 min and clarified by centrifugation. Protein concentrations were determined using the BCA Protein Assay kit (Pierce, Rockford, IL, USA). For Western blotting, equal amounts of protein samples were size-separated on 8% polyacrylamide gels and electroblotted onto a polyvinylidene difluoride (PVDF) membrane. Non-specific binding was blocked by immersion of the membranes for 0.5 h in 2% skim milk in Tris-buffered saline (TBS) containing 0.1% Tween (TBS-T) at room temperature. The membranes were washed with TBS-T, incubated for 1.5 h at room temperature with primary antibodies, washed again and incubated for 1 h with secondary antibodies. The antigen was detected using ECL Western blotting Detection Reagents (Amersham Bioscience) according to the manufacturer's instructions.

### *Oligonucleotide microarray analysis*

Oligonucleotide microarray (GeneChip Human Genome U133A Plus 2.0 Array; Affymetrix, Santa Clara, CA, USA) was applied to analyze the gene expression profile in BEAS-2B induced by TGF β1, adult lung, fetal lung, and skin. This array contained probe sets interrogating approximately 47 400 transcript clusters. Analysis was carried out according to the manufacturer's protocol.