

## Full-length article

## Decreased expression of human aquaporin-5 correlated with mucus overproduction in airways of chronic obstructive pulmonary disease<sup>1</sup>

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### Key words

aquaporin-5; mucin 5AC; mucin; mucus overproduction; chronic obstructive pulmonary disease

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### Abstract

**Aim:** To investigate the relationship between aquaporin-5 (AQP<sub>5</sub>) expression and mucus overproduction in the airways of Chinese patients with chronic obstructive pulmonary disease (COPD) and the correlation with pulmonary function change. **Methods:** Bronchial tissues were obtained from fiberoptic bronchoscopy and bronchial biopsy in West China Hospital from April to July 2004. Twenty-five patients were diagnosed as COPD patients, and another 20 were diagnosed as the control patients. The expressions of AQP<sub>5</sub>, mucin 5AC (MUC5AC), and mucin in bronchial tissues were detected by semiquantitative RT-PCR, *in situ* hybridization, and immunohistochemical and alcian blue-periodic acid-Schiff (AB-PAS) staining, respectively. **Results:** Compared with the control group, an attenuated expression of AQP<sub>5</sub> was detected throughout the bronchial tissues from patients with COPD ( $P < 0.01$ ), but no difference existed in the lung tissues ( $P > 0.05$ ). Simultaneously, increased staining of MUC5AC and mucus in submucosal glands were noted ( $P < 0.01$ , respectively). Smoking attenuated the expressions of AQP<sub>5</sub> and increased the staining of MUC5AC and mucus in submucosal glands in the COPD groups ( $P < 0.01$ ), while there were no significant differences observed in the control group ( $P > 0.05$ ). The decreased expression of AQP<sub>5</sub> mRNA was correlated with forced expiratory volume in 1 s (FEV<sub>1</sub>)/forced vital capacity (FVC) ( $r = 0.60$ ,  $P < 0.01$ ), FEV<sub>1</sub>% predicted value ( $r = 0.60$ ,  $P < 0.01$ ), maximal expiratory flow in 50% vital capacity ( $V_{50}$ )% predicted value ( $r = 0.55$ ,  $P < 0.01$ ), and maximal expiratory flow in 25% vital capacity ( $V_{25}$ )% predicted value ( $r = 0.45$ ,  $P < 0.01$ ). The decreased expression was negatively correlated with MUC5AC mRNA of the epithelium airways ( $r = -0.45$ ,  $P < 0.01$ ) and the AB-PAS-stained area of submucosal glands ( $r = -0.61$ ,  $P < 0.01$ ). The upregulation of MUC5AC mRNA correlated with the positively AB-PAS-stained area of submucosal glands and correlated negatively with FEV<sub>1</sub>/FVC ( $r = -0.53$ ;  $P < 0.01$ ), FEV<sub>1</sub>% predicted value ( $r = -0.53$ ;  $P < 0.01$ ),  $V_{50}$ % predicted value ( $r = -0.48$ ;  $P < 0.01$ ), and  $V_{25}$ % predicted value ( $r = -0.43$ ;  $P < 0.01$ ). **Conclusion:** The attenuated gene expression of AQP<sub>5</sub> existed in the airways of Chinese COPD patients, which was complicated by mucus hypersecretion. The decreased expression of AQP<sub>5</sub> mRNA may be related to the severity of airflow obstruction.

### Introduction

Water transport across epithelial and endothelial barriers

in bronchopulmonary tissues occurs during airway hydration, alveolar fluid transport, and submucosal gland secretion. Many of the tissues involved in these processes

are highly water permeable and express aquaporin (AQP) water channels<sup>[1]</sup>. AQP<sub>1</sub> is expressed in microvascular endothelia throughout the lung and airways, AQP<sub>3</sub> in basal epithelial cells in large airways and throughout the nasopharynx, AQP<sub>4</sub> at the basolateral plasma membrane surface in epithelia throughout the trachea and the small and large airways, and AQP<sub>5</sub> in type I alveolar epithelial cells and submucosal gland acinar cells<sup>[2]</sup>. In the lungs, AQP<sub>1</sub> and AQP<sub>5</sub> provide the principal route for osmotically-driven water transport<sup>[3]</sup>. AQP<sub>5</sub> deletion in submucosal glands in the upper airways reduced fluid secretion and increased protein content<sup>[4]</sup>.

Chronic obstructive pulmonary disease (COPD) is a major cause of chronic morbidity and mortality throughout the world, and is usually complicated by mucus hypersecretion in the airways leading to repeated infection<sup>[5]</sup> and irreversible airflow limitation<sup>[6]</sup>. Mucus consists mainly of water (95%) combined with salts, lipids, proteins, and glycoprotein. Abnormal mucus secretions have been proposed to promote bacterial adhesion and inhibit bacterial clearance by impeding cilia function<sup>[7]</sup>, which may lead to exacerbations in COPD patients. So far, studies about mucus are focused on the regulation of mucin synthesis. However, the secretion of mucin is intimately related to the secretion of water and ions as well, for which little study has been done.

Thirteen mucin genes have been discovered<sup>[8]</sup>. Mucin 5AC (MUC5AC), a secreted mucin, colocalizes with glycoprotein-positive goblet cells and submucosal gland mucus cells. Thus, MUC5AC is the marker of mucus production in the epithelium airways<sup>[9]</sup>. In this study, we hypothesized that an abnormal gene expression of AQP may exist and become complicated by mucus hypersecretion in the airways of COPD patients. To this end, we examined the expression levels of AQP<sub>5</sub>, MUC5AC, and mucin in bronchial tissues of Chinese COPD patients to investigate the relationship of AQP<sub>5</sub> and mucus overproduction.

## Materials and methods

**Bronchial tissues** Bronchial tissues obtained from fiberoptic bronchoscopy and bronchial biopsy in West China Hospital (Sichuan, China) between April and July 2004 were prepared as described<sup>[10]</sup>. Twenty-five patients were diagnosed as COPD patients according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) 2004 (<http://www.goldcopd.com>), and another 20 were the control patients without COPD. Patients with asthma, cystic fibrosis, bronchiectasis, and diffuse panbronchiolitis were excluded. The COPD patients had history of productive coughing for

3 consecutive months each year for the past 2 years, with a forced expiratory volume in 1 s (FEV<sub>1</sub>) <80% of the predictive value, a FEV<sub>1</sub>/forced vital capacity (FVC) ratio <70%, and a reversibility in FEV<sub>1</sub> <10% after inhalation of 400 µg salbutamol. According to FEV<sub>1</sub>% predicted value, there were 8 mild, 16 moderate, and 1 severe patient, respectively. The control patients had foreign body in the bronchus or benign bronchial stenosis and needed treatment with fiberoptic bronchoscopy. Eight healthy volunteers were also included in the control group (Table 1). All patients were ex-smokers or non-smokers, and the ex-smokers had given up smoking for more than 5 years. All patients had no respiratory tract infections within 2 weeks preceding the operation. The sites of biopsy were randomized to either the right or the left lower lobe, and 5 to 6 endobronchial biopsy specimens were taken from the tertiary carinae of the right or left lower lobes under direct visualization. The ethics committee of the West China Hospital, Sichuan University approved the study, and all patients gave written informed consent.

**Table 1.** Patients' data. <sup>a</sup>*P*>0.05, <sup>b</sup>*P*<0.05, <sup>c</sup>*P*<0.01 vs the control group; <sup>d</sup>*P*>0.05, <sup>e</sup>*P*<0.05, <sup>f</sup>*P*<0.01 vs the mild COPD group.

	Control group (n=20)	Mild COPD group (n=8)	Moderate-severe COPD group (n=17) <sup>f</sup>
Age (years)	53.9±10.5	57.4±5.3 <sup>a</sup>	61.4±9.2 <sup>ad</sup>
Sex (male/female)	12/8	5/3 <sup>a</sup>	10/7 <sup>ad</sup>
Smoking, NS/ES	10/10	3/5 <sup>a</sup>	5/12 <sup>ad</sup>
Smoking, pack-year	4.3±1.6	18.7±10.5 <sup>c</sup>	34.1±17.5 <sup>cf</sup>
FEV <sub>1</sub> /FVC	78%±6%	65%±4% <sup>c</sup>	59%±5% <sup>cf</sup>
FEV <sub>1</sub> % predicted value	79%±6%	65%±4% <sup>c</sup>	59%±5% <sup>ce</sup>
V <sub>50</sub> % predicted value	94%±24%	53%±17% <sup>c</sup>	44%±14% <sup>ce</sup>
V <sub>25</sub> % predicted value	96%±45%	39%±45% <sup>c</sup>	28%±16% <sup>cf</sup>

NS/ES, non-smoker/ex-smoker.

**RT-PCR** To examine the AQP<sub>5</sub> mRNA levels in the human airways, semiquantitative RT-PCR was performed. Total RNA was isolated with Trizol reagent in accordance with the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). The AQP<sub>5</sub> primer sequences were as follows: upstream, 5'-AAG GCC GTG TTC GCA GAG TT-3' and downstream, 5'-TGG TCA GCT CCA TGG TCT TC-3'. The MUC5AC primer sequences were as follows<sup>[11]</sup>: upstream, 5'-TCC GGC CTC ATC TTC TCC-3' and downstream, 5'-ACT TGG GCA CTG GTG CTG-3'. The GADPH primer sequences were as follows: upstream, 5'-GAG CGA GAT CCC TCC AAA-3' and downstream, 5'-ACT GTG GTC ATG AGT CCT TC-3'. The RT-PCR

reagents were purchased from Promega Corporation (Madison, MI, USA). The reaction mixture contained 10  $\mu$ L Avian Myeloblastosis Virus (AMV)/*Thermus flacus* (Tfl) 5 $\times$  reaction buffer, 0.2 mmol/L deoxy-ribonucleoside triphosphate (dNTP) mixture, 1  $\mu$ mol/L of each primer, 1 mmol/L  $MgSO_4$ , 5U AMV reverse transcriptase, 5U Tfl DNA polymerase, and 1  $\mu$ g RNA sample in 50  $\mu$ L volume. The first-strand cDNA was synthesized at 48 °C for 45 min, followed by denature of the template at 94 °C for 2 min. Amplification was achieved with 40 cycles of denaturing (94 °C, 30 s), annealing (57 °C, 1 min), and extension (68 °C, 2 min). The final extension was performed at 68 °C for 7 min. The PCR products were analysed by 1.5% agarose gel electrophoresis, and the expressions were measured by densitometry using Molecular Analysis Software (BioRad, Hercules, CA, USA). Relative quantitations of gene expressions were normalized to GAPDH with each sample.

***In situ* hybridization** The bronchial sections were fixed with 4% neutral buffered formaldehyde in 0.1% diethyl pyrocarbonate (DEPC). Six 4- $\mu$ m sections were cut from each bronchial tissue block. A digoxigenin-labeled 24 bp RNA probe was designed by Shenergy Biocolor Company (Shanghai, China). The AQP<sub>5</sub> primer sequence was 5'-AGA GCA GGT AGA AGT AAAGGATGG CAG-3'. The probe was diluted in 50 pmol/ $\mu$ L. ISH reagents were purchased from Pan Path (Amsterdam, the Netherlands). Bound probes were detected with alkaline phosphatase-conjugated anti-digoxin (DIG) antibodies, and Nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate was used as substrate. The sections were counterstained with methyl green. Hybridizations of several sections with AQP<sub>5</sub> sense probes were used as negative controls.

The samples were dewaxed in xylene and dehydrated in a graded ethanol series prehybridization. Tissues were incubated for 15 min at 37 °C in 1 mg/mL proteinase K, and then blocked for 10 min in 0.1 mol/L triethanolamine and 0.25% acetic solution before equilibration in 2 $\times$  standard saline citrate (SSC). The sections were prehybridized with 50% formamide in 2 $\times$ SSC for 4 h at 50 °C. Hybridization was carried out overnight at 42 °C and then the slides were washed for 15 min in 4 $\times$ SSC at 37 °C and exposed for 30 min to 10  $\mu$ g/mL RNase in 2 $\times$ SSC at 37 °C. After incubation for 30 min with normal goat blood serum, alkaline phosphatase-conjugated anti-DIG antibodies and Nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate were added as substrates. The sections were counterstained with methyl green.

**Histochemical analysis** The bronchial sections were fixed with 4% neutral buffered formaldehyde. Six 4- $\mu$ m sections

were cut from each bronchial tissue block. Antihuman AQP<sub>5</sub> polyclonal antibody and MUC5AC polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and diluted at 1:100. The sections were stained with diaminobenzidine (DAB).

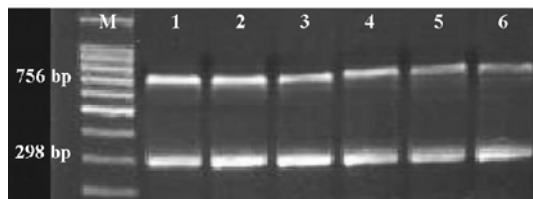
The samples were dewaxed in xylene and dehydrated in a graded ethanol series. Endogenous peroxidase activity was inhibited by incubating the slides with 3% H<sub>2</sub>O<sub>2</sub> for 30 min, followed by washing in phosphate-buffered solution (PBS) and microwave heating (700 W) for 15 min in 10 mmol/L citric acid (pH 6.0). The sections were inactivated with normal goat serum for 20 min at 37 °C. After repeated washing with PBS, the sections were incubated with the AQP<sub>5</sub> and MUC5AC antibodies, placed in a humid chamber at 4 °C overnight, washed in PBS, and incubated at 37 °C for 30 min with biotinylated rabbit anti-goat immunoglobulin, followed by repeated washing in PBS. The tissue was incubated with Streptavidin Peroxidase (SP) reagents for 30 min at 37 °C and then the sections were stained with freshly prepared DAB. After repeated washing with PBS and rinsing with tap water, the slides were counterstained with hematoxylin, and alcian blue-periodic acid-Schiff (AB-PAS) staining was performed as usual.

**Image analysis** All the images were generated by Spot Advanced Software (Diagnostic Instruments, MI, USA) and analyzed by IMAGE-Pro plus Version 4.5 (Media Cybernetics, HWY, USA). Two pathologists blinded to the patients' clinical data reviewed all the images and made the analysis. High-power fields of bronchial tissues were randomly sampled and calculations were performed on 6 images per section examined at  $\times$ 200 magnification. The mean of the 6 sections from each tissue was used for the statistical analysis. For the *in situ* hybridization and immunohistochemistry, the optical density (OD) of the positively-stained cells was normalized to the OD of the background in the same sections. For AB-PAS staining, the positively-stained areas in the submucosal glands were used to evaluate proliferations of the submucosal glands, normalized to areas of the bronchial tissues in the same fields of view.

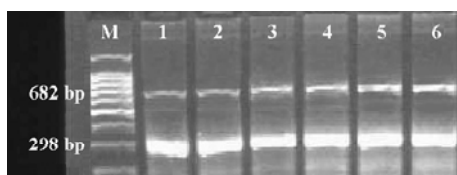
**Statistical analysis** All data were expressed as the mean  $\pm$  SEM. Statistical analyses were performed using SPSS 12.0 software (SPSS Inc, IL, USA). Statistical significance was analyzed with one-way ANOVA, followed by Student-Newman-Keuls test to isolate significant difference.  $\chi^2$ -test and the Pearson correlation analysis were also used. A *P*-value less than 0.05 (2-tailed test) was considered statistically significant.

## Results

**AQP<sub>5</sub> and MUC5AC mRNA expression in bronchial tissues** To investigate the expression of AQP<sub>5</sub> and MUC5AC mRNA, RT-PCR was performed. The signals of AQP<sub>5</sub> and MUC5AC were detected in the bronchial tissues of all the patients. Bands of AQP<sub>5</sub>, MUC5AC, and GAPDH PCR products were located at 756, 682, and 298 bp, respectively (Figure 1). Compared with the control group, attenuated expressions of AQP<sub>5</sub> were detected in the mild, moderate, and severe COPD groups ( $P<0.01$ ,  $P<0.01$ ). The mean OD of AQP<sub>5</sub> were  $0.68\pm 0.01$  in the control group,  $0.62\pm 0.03$  in the mild COPD group, and  $0.56\pm 0.04$  in the moderate and severe COPD group (Figure 1A). The upregulated expressions of MUC5AC were also found in 2 COPD groups ( $P<0.01$ ,  $P<0.01$ ). The mean OD of MUC5AC were  $0.43\pm 0.03$  in the control group,  $0.48\pm 0.05$  in the mild COPD group, and  $0.54\pm 0.04$  in the moderate



**Figure 1A.** Expression of AQP<sub>5</sub> in submucosal glands (RT-PCR). M was 100 bp DNA marker. 1 and 2 were the results of the control group. 3 and 4 were the results of the mild COPD group. 5 and 6 were the results of the moderate and severe COPD group. Bands of AQP<sub>5</sub> and GAPDH PCR products were located at 756 bp and 298 bp, respectively. Compared with the control group, attenuated expressions of AQP<sub>5</sub> was detected in both of the COPD groups ( $P<0.01$ ,  $P<0.01$ ). Furthermore, the expression of AQP<sub>5</sub> in the moderate and severe COPD patients was decreased compared with that in the mild COPD patients ( $P<0.01$ ).



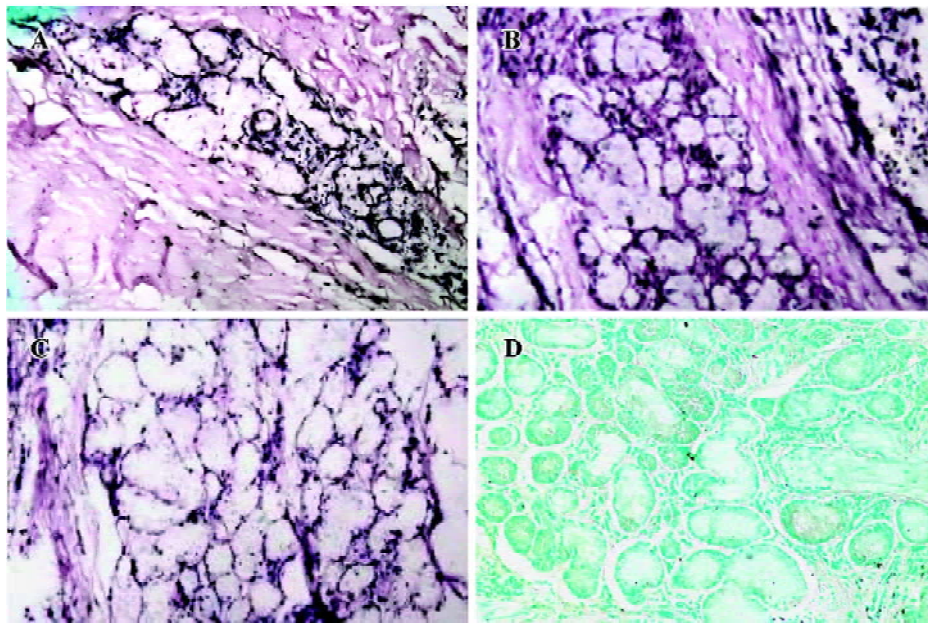
**Figure 1B.** Expression of MUC5AC in airway epithelial cells (RT-PCR). M was 100 bp DNA marker. 1 and 2 were the results of the control group. 3 and 4 were the results of the mild COPD group. 5 and 6 were the results of the moderate and severe COPD group. Bands of MUC5AC and GAPDH PCR products were located at 682 bp and 298 bp, respectively. Compared with the control group, increased expressions of MUC5AC was detected in the both COPD groups ( $P<0.01$ ,  $P<0.01$ ). Furthermore, the expression of MUC5AC in the moderate and severe COPD patients was increased compared with that in the mild COPD patients ( $P<0.01$ ).

and severe COPD group (Figure 1B). In the lung tissues, the differences in AQP<sub>5</sub> in the groups were not noted and there was no MUC5AC expression in the lung tissues in all the groups (data not shown).

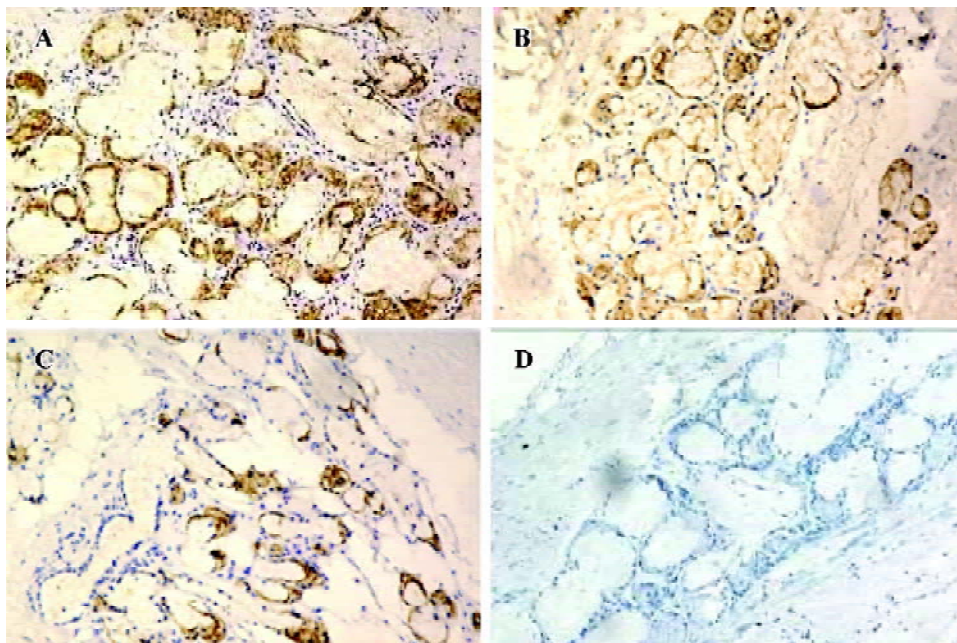
The influence of smoking on the expression of AQP<sub>5</sub> and MUC5AC mRNA was also evaluated. There were 17 ex-smokers and 8 non-smokers in the COPD group, and 10 ex-smokers and 10 non-smokers in the control group. In the COPD group, the mean OD of AQP<sub>5</sub> were  $0.58\pm 0.03$  for the ex-smokers and  $0.63\pm 0.04$  for the non-smokers, and the mean OD of MUC5AC were  $0.53\pm 0.04$  for the ex-smokers and  $0.49\pm 0.03$  for the non-smokers. Both differences were significant ( $P<0.05$ ,  $P<0.05$ ), but in the control group, the mean OD of AQP<sub>5</sub> was  $0.67\pm 0.03$  for the ex-smokers and  $0.68\pm 0.04$  for the non-smokers, and the mean OD of MUC5AC was  $0.45\pm 0.04$  for the ex-smokers and  $0.43\pm 0.03$  for the non-smokers, without significant differences ( $P>0.05$ ,  $P>0.05$ ).

To identify the cell-specific expression of AQP<sub>5</sub>, *in situ* hybridization was performed. AQP<sub>5</sub> expression was detected mainly in the submucosal gland cells, type I alveolar cells, and secondarily in the airway epithelial cells from all groups of patients. The positive signals (in purple) were expressed mainly in the nucleus and secondarily on the cell membrane. The COPD groups showed attenuated expressions and less positively-stained cells of submucosal glands and epithelial airway cells than the control group. The mean OD was  $1.37\pm 0.05$  in the control group,  $1.35\pm 0.02$  in the mild COPD patients, and  $1.31\pm 0.02$  in the moderate and severe COPD patients; the differences were significant ( $P<0.01$ ,  $P<0.01$ ; (Figure 2A–2C). The differences of AQP<sub>5</sub> expression in type I alveolar cells of all groups were not significant ( $P>0.05$ ,  $P>0.05$ ; data not shown). Hybridization with the sense probe revealed no staining, confirming the specificity of the assay (Figure 2D).

**AQP<sub>5</sub> and MUC5AC protein expression in bronchial tissues** To examine the correlation between AQP<sub>5</sub> expression and MUC5AC synthesis, immunohistochemical staining for AQP<sub>5</sub> and MUC5AC were performed. AQP<sub>5</sub> was expressed mainly in submucosal glands, type I alveolar cells, and secondarily at the apical membranes of cellula columno-epithelialis from patients in all the groups. The positive signals (in brown) were expressed mainly in the cytoplasm and secondarily on the cell membrane. The COPD groups showed attenuated expressions of AQP<sub>5</sub> and less positively-stained cells of submucosal glands and epithelial airway cells than the control group. The mean OD was  $1.36\pm 0.02$  in the control group,  $1.34\pm 0.01$  in the mild COPD patients, and  $1.30\pm 0.02$  in the moderate and severe COPD patients; the differences were significant ( $P<0.01$ ,  $P<0.01$ ; Figure 3A–3C). The differences



**Figure 2.** Expression of AQP<sub>5</sub> in submucosal glands (ISH×200). A was the control group, and B was the mild COPD group, and C was the moderate and severe COPD group. D was the hybridization with the sense probe instead of the digoxigenin (DIG)-labeled 24 bp RNA probe. The COPD groups showed decreased expressions and less positively stained cells of submucosal gland cells than the control group ( $P<0.01$ ,  $P<0.01$ ). Also, the expression of AQP<sub>5</sub> in the moderate and severe COPD patients was decreased compared with that in the mild COPD patients ( $P<0.01$ ).



**Figure 3.** Expression of AQP<sub>5</sub> in submucosal glands (IHC×400). A was the control group, and B was the mild COPD group, and C was the moderate and severe COPD group. D was the staining with PBS instead of AQP<sub>5</sub> antibody. The COPD groups showed increased expressions and more positively stained cells of submucosal gland cells than the control group ( $P<0.01$ ,  $P<0.01$ ). Also, the expression of AQP<sub>5</sub> in the moderate and severe COPD patients was decreased compared with that in the mild COPD patients ( $P<0.01$ ).

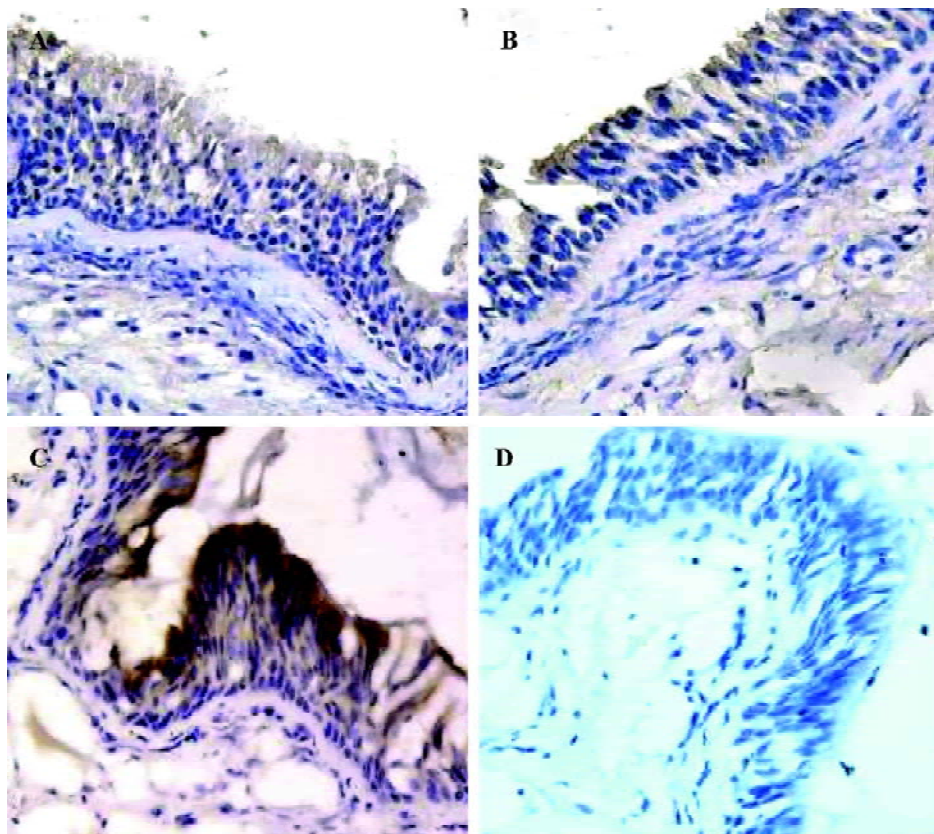
of AQP<sub>5</sub> expression in type I alveolar cells of all groups were not significant ( $P>0.05$ ,  $P>0.05$ ; data not shown).

MUC5AC was expressed throughout the bronchial tissues from patients in all groups, but no positive signals were found in the lung tissue of any group. The positive signals (in brown) were expressed mainly in the cytoplasm and secondarily on the cell membrane. The COPD groups showed stronger expression and more positively-stained cells than the control group. The mean OD was  $1.33\pm 0.01$  in the control group,  $1.37\pm 0.01$  in the mild COPD patients, and  $1.41\pm 0.03$  in moderate and severe COPD patients; the differences were significant ( $P<0.01$ ,  $P<0.01$ ; Figure 4A–4C). When PBS was used instead of the AQP<sub>5</sub> and MUC5AC antibodies, no staining resulted, confirming the specificity of the assay (Figures 3D,4D).

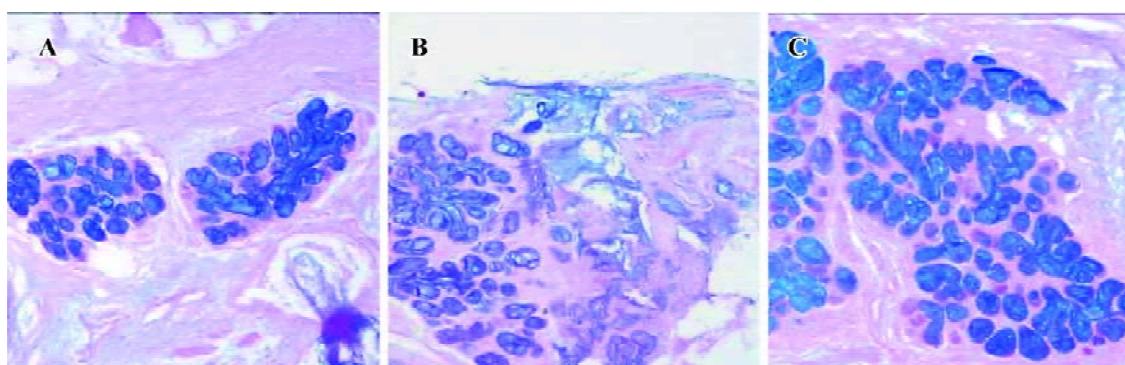
The results of the immunohistochemical staining for AQP<sub>5</sub> and MUC5AC were used to evaluate the influence of smoking. In the COPD group, the mean OD of AQP<sub>5</sub> was  $1.31\pm 0.02$  for the ex-smokers and  $1.34\pm 0.02$  for the non-

smokers, and the mean OD of MUC5AC was  $1.42\pm 0.02$  for the ex-smokers and  $1.37\pm 0.03$  for the non-smokers. Both differences were significant ( $P<0.05$ ,  $P<0.01$ ), but in the control group, the mean OD of AQP<sub>5</sub> was  $1.36\pm 0.03$  for the ex-smokers and  $1.35\pm 0.02$  for the non-smokers. The mean OD of MUC5AC was  $1.31\pm 0.02$  for the ex-smokers and  $1.34\pm 0.02$  for the non-smokers, without significant differences ( $P>0.05$ ,  $P>0.05$ ).

**Mucin production in bronchial tissues** To examine whether the decreased expression of AQP<sub>5</sub> was associated with mucus hypersecretion, AB-PAS staining were performed. The sialic acid in the submucosal gland acinar cells was stained blue, while polysaccharide and neutral mucin were stained purple. Submucosal glands in bronchial tissues from all groups produce mucin. The ratios of the positively-stained areas in the submucosal glands to the areas of bronchial tissues in the same fields of view were  $0.26\pm 0.01$  in the control group,  $0.31\pm 0.02$  in the mild COPD patients, and  $0.38\pm 0.04$  in the moderate and severe COPD patients; signifi-



**Figure 4.** Expression of MUC5AC in bronchial tissues (ICH $\times 400$ ). A was the control group, and B was the mild COPD group, and C was the moderate and severe COPD group. D was the staining with PBS instead of MUC5AC antibody. The COPD groups showed increased expressions and more positively stained cells of airway epithelial cells than the control group ( $P<0.01$ ,  $P<0.01$ ). Also, the expression of MUC5AC in the moderate and severe COPD patients was increased compared with that in the mild COPD patients ( $P<0.01$ ).



**Figure 5.** Mucin expression in submucosal glands (AB-PAS,  $\times 100$ ). A was the control group, and B was the mild COPD group, and C was the moderate and severe COPD group. The COPD groups showed increased expressions of mucins than the control group ( $P < 0.01$ ,  $P < 0.01$ ). Also, the expression of mucins in the moderate and severe COPD patients was increased compared with that in the mild COPD patients ( $P < 0.01$ ).

cant differences were noted ( $P < 0.01$ ,  $P < 0.01$ ; Figure 5A–5C).

Smoking resulted in increased positively-stained areas in the submucosal glands. In the COPD group, they were  $0.37 \pm 0.02$  for the ex-smokers and  $0.32 \pm 0.02$  for the non-smokers,  $0.25 \pm 0.01$  for the ex-smokers and  $0.26 \pm 0.01$  for the non-smokers in the control group. The difference in the COPD group was significant ( $P < 0.01$ ), and that of the control group was to the contrary ( $P > 0.05$ ).

**Correlation analysis** The downregulation of AQP<sub>5</sub> expression correlated significantly with mucus overproduction, the data of FEV<sub>1</sub>/FVC and FEV<sub>1</sub>% predicted values, V<sub>50</sub>% predicted value, and V<sub>25</sub>% predicted value. The expression levels of MUC5AC mRNA negatively correlated with the data of FEV<sub>1</sub>/FVC and FEV<sub>1</sub>% predicted values, V<sub>50</sub>% predicted value, and V<sub>25</sub>% predicted value (Table 2).

## Discussion

The discovery of the AQP family of water channel proteins has provided insight into molecular mechanisms of membrane water permeability. The specific expression pattern of AQP<sub>5</sub> in the lungs suggests that it plays a role in the regulation of mucus secretion. In this study, we evaluated

the expression of AQP<sub>5</sub>, MUC5AC, and mucin in the airways in bronchial tissues. Attenuated AQP<sub>5</sub> mRNA expression was demonstrated in the airways of COPD patients. Concomitantly, MUC5AC and mucus secretion increased greatly in the airways of COPD patients. Smoking attenuated the expressions of AQP<sub>5</sub> and increased the staining of MUC5AC and mucin in submucosal glands in the COPD groups, while there were no significant differences in the control group. The downregulation of AQP<sub>5</sub> expression correlated significantly with mucus overproduction, the data of FEV<sub>1</sub>/FVC and FEV<sub>1</sub>% predicted values, V<sub>50</sub>% predicted value, and V<sub>25</sub>% predicted value. The expression levels of MUC5AC mRNA negatively correlated with the data of FEV<sub>1</sub>/FVC and FEV<sub>1</sub>% predicted values, V<sub>50</sub>% predicted value, and V<sub>25</sub>% predicted value. These results suggest that the attenuated gene expression of AQP<sub>5</sub> existed and was complicated by mucus hypersecretion in the airways of Chinese COPD patients. Smoking results in airway remodeling in patients with COPD.

Mucus protects the underlying airway epithelium from dehydration, pathogens, and chemical and particulate irritants, but mucus hypersecretion in the airways leads to the obstruction of airflow. Abnormal mucus secretions have been proposed to promote dysfunction of antibiosis enzymes

**Table 2.** Correlation analysis. <sup>b</sup> $P < 0.05$ , <sup>c</sup> $P < 0.01$ .

	FEV <sub>1</sub> /FVC	FEV <sub>1</sub> % predicted value	V <sub>50</sub> % predicted value	V <sub>25</sub> % predicted value	AB-PAS	MUC5AC mRNA
AQP <sub>5</sub> mRNA ( <i>r</i> )	0.6 <sup>c</sup>	0.6 <sup>c</sup>	0.55 <sup>c</sup>	0.45 <sup>c</sup>	-0.61 <sup>c</sup>	-0.45 <sup>c</sup>
MUC5AC mRNA ( <i>r</i> )	-0.53 <sup>c</sup>	-0.53 <sup>c</sup>	-0.48 <sup>c</sup>	-0.43 <sup>c</sup>	0.43 <sup>b</sup>	

and antibacterial peptides<sup>[7]</sup>. Submucosal glands are thought to play an important role in the regulation of mucus volume and composition, and are important in antimicrobial defense<sup>[12]</sup>. Immunocytochemistry of human and rat airways indicated the expression of AQP water channels in glandular epithelia<sup>[13,14]</sup>. In the current study, we found the expression of AQP<sub>5</sub> at the apical membranes of cellular columnoepithelialis and submucosal glands, the sites of active mucus secretion. This confirmed the importance of AQP<sub>5</sub> in the regulation of mucus secretion. AQP<sub>5</sub> was also found expressed in the apical surface of type I pneumocytes<sup>[15]</sup>, which involved fluid movement across epithelial and endothelial barriers occurring in interstitial and alveolar pulmonary edema. Song *et al*<sup>[16]</sup> showed that gland fluid from AQP<sub>5</sub>-null mice had a substantially higher protein concentration and mildly elevated chloride concentration compared with fluid from wild-type mice. Ma *et al*<sup>[17]</sup> found that AQP<sub>5</sub> deletion resulted in impaired secretion of fluid from salivary glands. Furthermore, Krane *et al*<sup>[18]</sup> showed that the decreased rate of fluid secretion onto the airway surface in AQP<sub>5</sub>-null mice could increase airway reactivity in response to bronchoconstricting agents. We found for the first that the expression of AQP<sub>5</sub> was attenuated throughout the bronchial tissues from patients with COPD. Moreover, the attenuated expression of AQP<sub>5</sub> was related to the severity of airflow obstruction. Thus, AQP<sub>5</sub> deletion may be one of the causes of hypertonic mucus and airway hyperresponsiveness in patients with COPD.

Smoking is a main factor in the pathogenesis of COPD. We evaluated the influence of smoking on the expressions of AQP<sub>5</sub>, MUC5AC, and mucus in submucosal glands of ex-smokers and non-smokers in the control and COPD groups, respectively. Interestingly, smoking attenuated the expressions of AQP<sub>5</sub> and increased the staining of MUC5AC and mucin in submucosal glands in the COPD group, while there were no significant differences in the control group. Although all ex-smokers had given up smoking for more than 5 years, the influence of smoking continued to exist in patients with COPD. This might be the result of airway remodeling in these patients, but it seemed that smoking did not play any role in the control group. Only 15% of cigarette smokers develop clinically-significant COPD<sup>[19]</sup>. Whether AQP<sub>5</sub> gene polymorphisms are involved in the pathogenesis of COPD needs further investigation.

In COPD patients, the impaired hypertonic mucus by AQP<sub>5</sub> deletion may be a functional rather than anatomical defect as we found that greater areas of submucosal glands were noted in the COPD group than in the control group, and the expression levels of AQP<sub>5</sub> mRNA significantly correlated with the patients' data of FEV<sub>1</sub>/FVC and FEV<sub>1</sub>% predicted values,

V<sub>50</sub>%, predicted value, and V<sub>25</sub>% predicted value. The AQP<sub>5</sub> deletion and MUC5AC increase may reflect, at least in part, the airflow obstruction. Towne *et al*<sup>[20]</sup> showed that tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) inhibited the expression of AQP<sub>5</sub> mRNA and protein in mouse lung epithelia cells. Recently, Lora *et al*<sup>[21]</sup> demonstrated that TNF- $\alpha$  triggered mucus production in the epithelium airways through an I $\kappa$ B kinase  $\beta$ -dependent mechanism. So TNF- $\alpha$  may regulate mucus secretion in 2 ways. In a cultured human airway epithelial cell line, the expression of AQP<sub>3</sub> was upregulated with the treatment of corticosteroid<sup>[22]</sup>, although the use of corticosteroids in stable COPD patients is still controversial. It will be helpful to investigate the possible regulators, including TNF- $\alpha$  and corticosteroid, for the gene expression of AQP<sub>5</sub>, MUC5AC, and mucin secretion in the epithelium airways of COPD patients.

In summary, the current study demonstrates that attenuated gene expression of AQP<sub>5</sub> existed and was complicated by mucus hypersecretion in the airways of Chinese COPD patients. The results suggest the importance of AQP<sub>5</sub> for fluid secretion of submucosal gland airways. Treatment targeting AQP<sub>5</sub> may be an effective approach to COPD therapy.

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