

# Polymorphisms of aquaporin5 gene in chronic obstructive pulmonary disease in a Chinese population

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## Summary

**Background:** Chronic obstructive pulmonary disease (COPD) is a major cause of chronic morbidity and mortality. It is influenced by both environmental and genetic factors. Aquaporin5 plays a critical role in the maintenance of normal lung water homeostasis. We investigated whether polymorphisms in the gene aquaporin5 had any bearing on individual susceptibility to the development of COPD.

**Methods:** 332 COPD patients and 373 unrelated, age-matched healthy people were recruited for the study. All participants were Chinese Han people. We designed denaturing high performance liquid chromatography (DHPLC) to detect SNP in exons1, 2, 3 of AQP5 and DNA sequencing to confirm it. The allele +2254A>G (rs3736309) in intron3 and +3088A>G (Thr-Thr, rs41308104) in exon4 from NCBI dbSNP were genotyped by PCR-based restriction fragment length polymorphism.

**Results:** We found no SNPs in exons1–4 of the AQP5 gene in our study population. However, the genotype frequency of +2254A>G SNP in intron3 was significantly different in cases and controls. The frequencies of AA AG GG in cases were 45.2%, 40.7%, and 14.1%, while in controls they were 34.5%, 51.6%, and 13.9% ( $\chi^2 = 9.899$ ,  $P = 0.007$ ), respectively. Higher OR for COPD was seen for persons with +2254AA genotype against +2254AG genotype (OR = 2.73; 95% CI = 1.88–3.97). Carriers of the variant allele +2254G had a lower risk of COPD than homozygous wild type carriers (OR = 0.44; 95% CI = 0.307–0.631).

**Conclusion:** The +2254A to G variant in intron 3 of AQP5 was associated with a decreased risk of COPD in a Chinese population.

**Key words:** aquaporin5; chronic obstructive pulmonary disease; single nucleotide polymorphism; intronic mutation

## Introduction

Chronic obstructive pulmonary disease (COPD) remains a major public health problem. It is a preventable and treatable disease state characterized by airflow limitation that is not fully reversible. COPD is an important and growing cause of morbidity and mortality worldwide. The WHO Global Burden of Disease Project estimated that COPD was the fifth leading cause of death worldwide in 2001 and would be the third leading cause by 2020 [1–2]. It is also an increasingly common problem in China. The prevalence of COPD in adults aged  $\geq 40$  years was 8.2% according to the survey among 20,245 participants in seven regions in China in 2007 [3].

Tobacco smoking continues to be a major cause of COPD. However, only 10–20% of heavy cigarette smokers develop COPD [4], which sug-

gests that genetic factors are also involved in determining individual susceptibility [5]. Epigenetic studies have gained increasing attention in the exploration of underlying causes for different susceptibilities. So far, more and more candidate genes have been identified as being related to COPD, including proteases–antiproteases, oxidases–antioxidases, xenobiotic biotransformation

### Abbreviations

AQP5	aquaporin5
COPD	chronic obstructive pulmonary disease
DHPLC	denaturing high performance liquid chromatography
RFLP	restriction fragment length polymorphism
SNP	single nucleotide polymorphism
GOLD	Global Initiative for Chronic Obstructive Lung Disease

enzymes, inflammatory mediators, cytokines and chemokines, genes relative to cell signal transduction pathway and others [6–13].

Most COPD patients suffer from mucus hypersecretion, which is one of the most important pathological and physiological characteristics in pulmonary obstructive diseases. There is significant association between airway hypersecretion and death among COPD patients [14]. Mucin viscosity is regulated by the movement of water, bicarbonate and glutathione, and a decrease in any of these factors increases the viscosity of mucin [15].

Aquaporins are water channel proteins that allow water to move rapidly through the plasma membrane in response to osmotic/hydrostatic pressure gradients [16]. Aquaporin5 (AQP5), a subtype of aquaporin family, is involved in fluid secretion of airway submucosal gland [17] and

modulation of mucin expression and secretion [18]. In a previous study an attenuated expression of AQP5 was detected throughout bronchial tissue from COPD patients compared with the healthy controls [19].

All these findings suggest that AQP5 may play a critical role in the maintenance of normal lung water homeostasis and changes in the amount of AQP5, and/or its function may contribute to abnormal water metabolism in various lung diseases. AQP5 gene was mapped to chromosome 12q13 and included 4 exons and 3 introns. It is assumed that gene variation is likely to affect the expression of AQP5 or its function. In the present study, we investigated genetic polymorphism of the AQP5 gene in order to explore the association of the polymorphisms with COPD susceptibility and thereby to predict the occurrence of the disease.

## Materials and methods

### Study subjects

332 COPD subjects were recruited from West China Hospital of Sichuan University. The confirmative diagnosis of COPD was based on the subject's medical history, spirometric data and post-bronchodilator FEV<sub>1</sub>/FVC. The cumulative cigarette dose (pack-year) was calculated using the following formula: pack-year = (packs per day) × (years smoked). Pulmonary function tests (CHESTAC-33-8800, Japan) were performed to determine FVC, FEV<sub>1</sub>, FEV<sub>1</sub>%predicted, FEV<sub>1</sub>/FVC and post-bronchodilator FEV<sub>1</sub>/FVC. Inclusion criteria for COPD patients were based on the diagnosis standards of COPD according to the American Thoracic Society (ATS) with post-bronchodilator FEV<sub>1</sub>/FVC <70% [20]. Patients with poorly reversible airflow limitation associated with bronchiectasis, cystic fibrosis and fibrosis due to tuberculosis were excluded. COPD Staging was based on post-bronchodilator spirometry according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) ([http://www.goldcopd.dk/index\\_uk.htm](http://www.goldcopd.dk/index_uk.htm) updated 2007). 373 unrelated, age-matched healthy people with normal pulmonary function, who had no known medical illness or family disorders, were chosen as control subjects. The control group was recruited from those patients visiting the same hospital for a health check-up or from community volunteers. Informed consent for participation in the study was obtained from each participant and approved by the Ethics Committee of West China Hospital, Sichuan University. Both the COPD subjects and controls were Chinese Han people.

### DNA extraction, screening of SNP in exons1, 2 and 3 by DHPLC

Genomic DNA samples for all subjects were extracted from EDTA-blood by Chelex-100 (Bio-Rad). PCR-based DHPLC analysis was used to search for novel variations in exons1, 2 and 3. Exon1 was divided into three overlapping fragments to be amplified by PCR. All primers, annealing temperatures and sizes of the PCR products are presented in table 1. The primers were designed according to the human genome sequence deposited in Genbank (GeneID: 362). PCR reaction mixture, 25 mL, contained 1.25 U of Pfu DNA polymerase

(BioTeke), 1× assay buffer, 10 pmol of each primer (Invitrogen), 0.2 mmol/L of dNTPs and about 50 ng DNA. PCR was set up in MyCycler™ thermal cycler (Bio-Rad). The amplicons of exons1, 2, and 3 were screened and scanned using DHPLC for novel SNPs. Prior to DHPLC analysis, heteroduplex formation was performed by 5 min denaturing at 96 °C and gradually renaturing over 46 cycles, decreasing to 1 °C at each cycle. The analysis was carried out on an automated HPLC device equipped with a DNA separation column (Wave System; Transgenomic, Omaha, NE, USA) using the conditions determined by the Transgenomic Wavemaker software. Samples that presented an elution profile with two peaks were checked by DNA sequencing from both directions.

### Genotyping of +2254A>G allele in intron3 and +3088A>G allele in exon4 by PCR-RFLP

The PCR product amplified by the primers of exon3 included the entry +2254A>G (rs3736309) in intron3 in the public single nucleotide polymorphism database (dbSNP). Primers for exon4 (table 1) were used to amplify the synonymous mutation +3088A>G (Thr-Thr, rs41308104) from dbSNP. The +2254A>G allele was genotyped by PCR-based *Xba* I (MBI) restriction fragment length polymorphism (PCR-RFLP). *Xba* I digestion cleaves the 337 bp PCR products into two fragments of 106 bp and 231 bp when A allele is present. The SNP +3088A>G was also analyzed by RFLP using *Nde* I enzyme (MBI) with A allele cleaved instead of G allele. The PCR products were digested according to the manufacture protocol. Restriction fragments were distinguished on 6% polyacrylamide gel and visualized by silver staining to determine the genotypes.

### Statistical analysis

SPSS13.0 software was used to perform the statistical analysis. Clinical data were presented as mean ± SD. The frequency of the genotype was counted. The cases and controls were compared using Student's t-test with 2-tailed values for the continuous variables and Chi-squared test for the categorical variables. Hardy-Weinberg equilibrium was tested for a goodness-of-fit Chi-square test with one degree of freedom to compare

the observed genotype frequencies among the subjects with the expected.

Odds ratios (OR) and 95% confidence interval (CI) of the genotype were calculated by age, gender and pack

years of smoking adjusted logistic regression analysis to quantify the association between the genotypes and COPD. Statistical significance was accepted at  $P < 0.05$ .

**Table 1**

Primers used for amplification of exons in AQP5 gene.

Region	Forward primers 5'-3'	Reverse primers 5'-3'	Annealing temperature (T <sub>m</sub> ) (°C)	Length of amplicons (bp)
Exon1	GGACCAAAGCCCGAAGA	TGTAGACCGGCCAGCGT	62	206
	CCAACCTGGGCGCATCAT	CCAAGAAGTCTGCGAACACG	64	488
	CGGCCACCATGAAGAAGGAG	TTTCCAGGTGGGGTAAGAGGC	63	448
Exon2	GGTGCCAAGGTGCTCTAAG	GCCCAATGTTCGGTACTGCT	61	295
Exon3	TGTGGGAGTGGACCAGGAT	GGTTCAGGCCATACAAAGC	60	337
Exon4	GGCTGCCATCCTTACTTCT	TGGTCTTCTCCGCTCTTC	61	131

## Results

### Clinical characteristics

A total of 705 subjects, including 332 COPD patients and 373 healthy controls, were studied. The demographic characteristics and relevant clinical parameters of all participants in the study are summarized in table 2. The mean age of the COPD patients, consisting of 225 men and 107 women, was 69 years old, compared with 67 years of the controls, consisting of 249 men and 124 women. No significant differences in sex, age or smoking history were observed between the COPD cases and controls. The parameters of Body Mass Index (BMI), FEV<sub>1</sub>, FVC, FEV<sub>1</sub>% predicted and FEV<sub>1</sub>/FVC were significantly decreased in the COPD patients compared with the controls ( $P = 0.000$ ).

### SNP screening by DHPLC

SNP screening of exons1, 2 and 3 of both COPD and controls by DHPLC showed there were no heteroduplex peaks except the amplicons of exon3. We selected different elution profiles (fig. 1) randomly to sequence. Results showed there was no SNP in exon3. The heteroduplex peaks turned out to be in intron3 according to the results of DNA sequencing (fig. 2) and BLAST search in NCBI.

### Distributions of +2254A>G (rs3736309) genotype in intron3

The genotype distributions of +2254A>G in controls and COPD patients are shown in table 3. There was no significant deviation in the genotype frequency from the Hardy-Weinberg equilibrium ( $P > 0.05$ ). The frequency of homozygote AA genotype was higher in COPD (45.2%) than in controls (34.5%). The heterozygote AG was, however, more frequent in the controls (51.6%) than in the cases (40.7%). The frequencies of genotypes +2254AA AG GG in cases were significantly different from those of controls ( $\chi^2 = 9.899$ ,  $P = 0.007$ ). In the logistic regression, individuals with +2254AA genotype were at 2.73-fold higher risk of COPD than heterozygous carriers (95% CI = 1.88–3.97). Carriers of the variant allele +2254G (AG and GG) had a lower risk of COPD than homozygous wild type carriers (OR = 0.44; 95% CI = 0.307–0.631) (table 3). No significant association was established between genotype distributions of +2254AA AG GG and spirometric classification of severity of COPD ( $P = 0.406$ ) (table 4).

### Polymorphisms of +3088A>G in exon4

The synonymous mutation allele +3088A>G (Thr-Thr, rs41308104) from NCBI dbSNP in

**Table 2**

Characteristics of the study population.

Variables	Control	COPD	P values (2-tailed)
Subjects (n)	373	332	–
Gender, M/F (n)	249/124	225/107	0.774
Age (years)	67.85 ± 9.16	69.35 ± 9.86	0.063
Smoke (pack-year)	19.04 ± 22.95	20.46 ± 27.92	0.572
BMI (kg/m <sup>2</sup> )	23.28 ± 3.32	21.05 ± 1.85	0.000
FEV <sub>1</sub> observed (L)	2.61 ± 0.44	1.45 ± 0.74	0.000
FVC observed (L)	3.15 ± 0.51	2.70 ± 0.83	0.000
FEV <sub>1</sub> % predicted	95.73 ± 11.22	53.82 ± 19.70	0.000
FEV <sub>1</sub> /FVC (%)	83.02 ± 6.80	53.24 ± 12.53	0.000

n: number of subjects

Data are presented as mean ± SD.

pack-year: (packs per day) × (years smoked)

BMI: Body Mass Index.

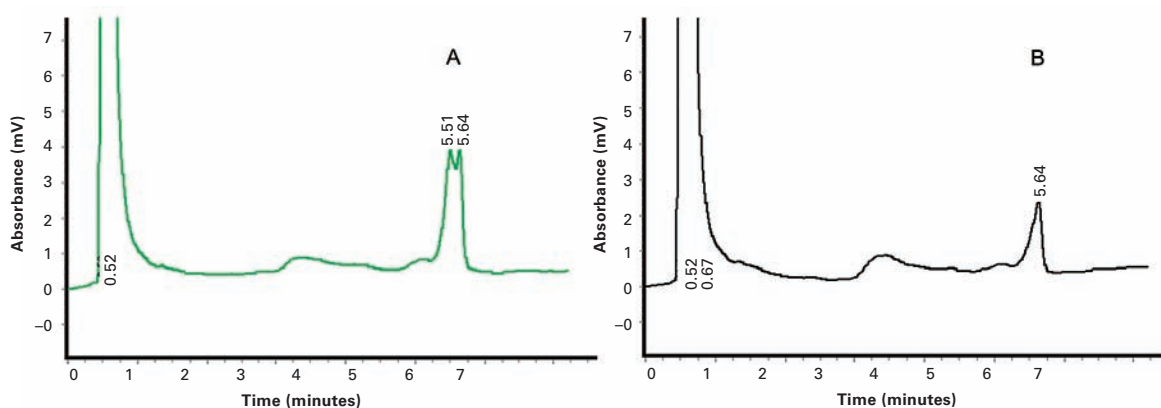
FEV<sub>1</sub>: forced expiratory volume in one second

FVC: forced vital capacity

**Figure 1**

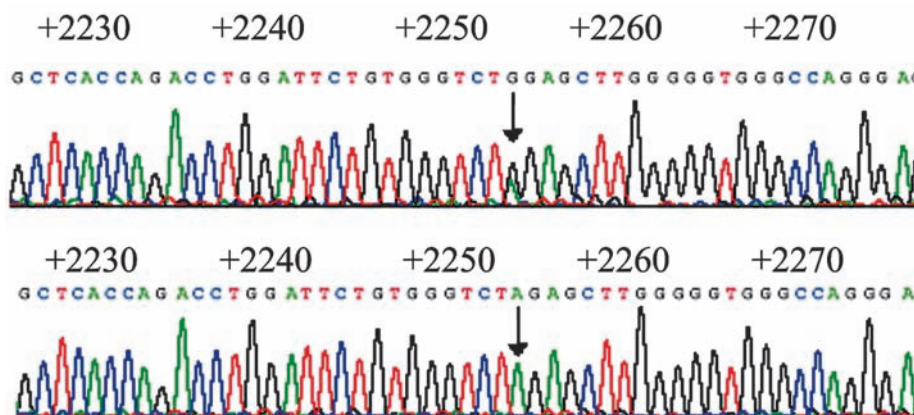
The DHPLC elution profile of exon3 amplicons with different elution peaks.

A: double elution peaks (heteroduplex peaks) indicated a heterozygote at a locus  
 B: single elution peak indicated a homozygote at a locus



**Figure 2**

Partial results of DNA sequencing of exon3 amplicons. The arrows showed heterozygote AG (above) and homozygote AA (below) in the DNA sequence.



**Table 3**

Genotype distribution of +2254A>G SNP in controls and COPD patients.

Polymorphism	Genotype distribution		Logistic regression analysis		
	Controls n (%)	COPD n (%)	OR	95%CI	P value
+2254AA	128 (34.5)	150 (45.2)	Reference		
+2254AG	193 (51.6)	135 (40.7)	0.366	0.252–0.532	0.000
+2254GG	52 (13.9)	47 (14.1)	1.146	0.617–2.129	0.667
+2254AG+GG	245 (65.5)	182 (54.8)	0.440	0.307–0.631	0.000

n: number of subjects  
 %: frequency of the genotype

**Table 4**

Spirometric classification of COPD severity based on post-bronchodilator FEV<sub>1</sub> according to GOLD criteria.

Genotype	Stage I n (%)	Stage II n (%)	Stage III n (%)	Stage IV n (%)	Chi-Square	P value (2-sided)
AA	23 (15.33)	53 (35.33)	50 (33.33)	24 (16.0)	6.156	0.406
AG	19 (14.07)	60 (44.44)	44 (32.59)	12 (8.89)		
GG	4 (8.51)	20 (42.55)	18 (38.30)	5 (10.64)		

GOLD: global initiative for chronic obstructive lung disease  
 Stage I: Mild FEV<sub>1</sub>/FVC <0.70, FEV<sub>1</sub> ≥80% predicted  
 Stage II: Moderate FEV<sub>1</sub>/FVC <0.70, 50% ≤FEV<sub>1</sub> <80% predicted  
 Stage III: Severe FEV<sub>1</sub>/FVC <0.70, 30% ≤FEV<sub>1</sub> <50% predicted  
 Stage IV: Very Severe FEV<sub>1</sub>/FVC <0.70, FEV<sub>1</sub> <30% predicted or FEV<sub>1</sub> <50% predicted plus chronic respiratory failure

exon4 turned out to be all homozygote AA genotypes in the cohort we studied, and no G allele appeared. This is different from that in Caucasian

asthmatic patients, as reported in the gene bank ([http://www.ncbi.nlm.nih.gov/SNP/snp\\_viewTable.cgi?pop=4539](http://www.ncbi.nlm.nih.gov/SNP/snp_viewTable.cgi?pop=4539)).

## Discussion

COPD is a complex disease and is influenced by the actions of multiple genes [21]. Its physiological characteristic changes include airflow limitation, mucus hypersecretion, air trapping and gas exchange abnormalities. In the peripheral lung and airways, fluid movement between the air-space, cellular/interstitial and vascular compartments is very important in the maintenance of air-space hydration and the secretion of mucus onto the airway surface by submucosal glands [22]. In healthy individuals, airway mucous layer is a defensive barrier against environmental stimuli. Inhaled particles are removed from the airway through mucus-cilia interaction. Under pathophysiological conditions such as asthma and COPD, excessive production of mucus results in airway obstruction and hyperresponsiveness, leading to exacerbation of these diseases. Major components of mucus include water, electrolytes, lipids, lactoferrin, lysozyme and mucin [23], in which water accounts for 95% [24] and AQP5 affects osmotic water permeability in the lung [25].

In this study, we investigated AQP5 polymorphisms in COPD in a case-control study. DHPLC, a robust technique enabling rapid, sensitive and accurate detection of SNPs in a large population, was performed to detect novel SNPs in exons of the AQP5 gene. The sensitivity and the specificity of DHPLC have been reported to range from 96% to 100% [26].

PCR-based RFLP was performed to genotype the SNP +2254A>G in intron3. To avoid a false positive result produced by incomplete digestion, it was further corroborated by DHPLC analysis of 20% heterozygote samples, and the results were consistent. The AG genotype of +2254A>G in intron3 was negatively associated with COPD, and allele G functioned as a protective factor for COPD. As was reported, the fact that introns are spliced out from gene transcripts does not imply that they cannot be subject to selection for their information or function. Some intronic sequences can be spliced out of some transcripts but can form part of the coding region in alternatively spliced transcripts. Some introns play regulatory roles in transcription or translation processes. Moreover, introns can also be involved in the maintenance of secondary structure of immature

messenger RNA (pre-mRNAs) [27]. Therefore, it remains to be elucidated whether the +2254A>G polymorphism itself affects gene function by affecting the splice donor site and/or regulatory motifs within the intron or its linkage disequilibrium with another polymorphism altering the function of AQP5. Alternatively, the decreased risk of COPD could be due to linkage equilibrium with another adjacent susceptible gene.

Previous studies have shown that the expression of AQP5 is related to the severity of airflow obstruction in COPD patients [19]. In this study we hypothesized that genotypes of +2254A>G in intron3 of AQP5 affected lung function in COPD patients, but no significant association was established between the genotypes and the stable stage pulmonary function of the patients. As longitudinal data are superior to cross-sectional, whether the genotypes of +2254A>G correlate with the decline of pulmonary function needs further study.

In conclusion, we found no SNPs in exons1-4 of the AQP5 gene in Chinese. However, the genotype frequency of +2254A>G SNP in intron3 was significantly different in cases and controls. The +2254A>G variant was associated with decreased risk of COPD. The finding deserves to be tested in a larger cohort and in diverse ethnic population. To our knowledge, this is the first molecular epidemiologic study of water channel protein polymorphism in COPD and is an addition to the previously published work on polymorphisms in the genes involved in the molecular pathophysiology of COPD. Our results may be helpful to predict the occurrence probability of the disease.

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