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Association of the *CYP1A1 Msp*I and TNFα-308 polymorphisms with chronic obstructive pulmonary disease in Inner Mongolia

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ABSTRACT. Chronic obstructive pulmonary disease (COPD) is a progressive lung disease characterized by persistent airflow limitation. Smoking, occupational exposures, air pollution, and genetics are all risk factors. In the present study, we detected the cytochrome P4501A1 gene (*CYP1A1*) *Msp*I polymorphism and the tumor necrosis factor alpha (TNFα)-308 single nucleotide polymorphism in COPD patients, and investigated their associations with smoking and COPD susceptibility in Inner Mongolia. A total of 101 COPD patients and 80 controls were enrolled in the study. The polymorphisms were analyzed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). *CYP1A1 Msp*I allele frequencies were significantly different between COPD patients and controls (P = 0.033). COPD susceptibility was higher in subjects with the m2 allele compared to subjects with the m1 allele [odds ratio (OR) = 2.531, 95% confidence interval (CI) = 1.297-4.940, P = 0.006]. Significant differences were observed in the TNFα-308

genotype and allele distributions between COPD patients and controls (P = 0.006 and P = 0.003, respectively). Compared to subjects with the GG genotype, subjects with GA+AA genotypes had higher COPD risk (OR = 3.639, 95%CI = 1.576-8.403, P = 0.002). The TNFα-308 polymorphism differed between smoking and non-smoking COPD patients and controls (P = 0.047 for genotype and P = 0.030 for allele). In conclusion, the *CYP1A1 Msp*I and TNFα-308 polymorphisms were associated with COPD susceptibility. Furthermore, of the two polymorphisms, only TNFα-308 may exert an interaction with smoking.

Key words: CYP1A1; TNFα; Polymorphism; Chronic obstructive pulmonary disease

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a lung disease characterized by persistent airflow limitation that is usually progressive over time. COPD is the fourth leading cause of death worldwide, and the third in the United States of America. The economic burden of COPD in 2007 was $42.6 billion, with respect to health care costs and loss of productivity. In China, COPD causes approximately 10% of deaths in urban areas, and 20% of deaths in rural areas (Qiao and Li, 2010). Tobacco smoking is the most common risk factor associated with COPD, with approximately 15% of smokers developing COPD (Fletcher and Peto, 1977). Non-smokers may also develop COPD, as occupational exposure, air pollution, and genetics are other risk factors. Deficiency of α-1-antitrypsin is a recognized genetic risk factor contributing to the disease. Protease-antiprotease imbalance, oxidative stress, and inflammation have all been put forward as hypotheses for the pathogenesis of COPD. These mechanisms may act separately or in concert in the development of COPD.

The cytochrome P450 (CYP450) enzyme system family is a primary metabolic pathway for the metabolism of intrinsic and extrinsic compounds such as carcinogens and drugs (Gardiner and Begg, 2006). CYP450 genes are highly polymorphic, and mutations in different genes can result in altered, reduced, increased, or no enzyme activity or changes in the amount of enzymes (Zhou et al., 2009). CYP1A1 metabolizes several procarcinogens, including polycyclic aromatic hydrocarbons (PAHs) (Guengerich and Shimada, 1998). Smoking, air pollution, and certain occupations are notable sources of PAHs, and are risk factors for COPD (Butler et al., 1993). Several polymorphisms have been identified in *CYP1A1*; however, a thymine (T) to cytosine (C) substitution at the *Msp*I site in the 3'-untranslated region (rs4646903) is commonly observed in Asian subjects. Although several studies have investigated the effect of the *CYP1A1 Msp*I polymorphism on lung diseases, such as lung cancer and COPD, no clear consensus has yet been reached (Houlston, 2000; Cheng et al., 2009; Ji et al., 2012).

The tumor necrosis factor alpha (TNFα) is a proinflammatory cytokine produced by activated macrophages, and can induce other inflammatory cytokines, chemokines, and growth factors (Wouters et al., 2009). Therefore, TNFα is thought to play a key role in the pathogenesis of COPD through tissue damage and remodeling (Churg et al., 2002; Mukhopadhyay et al., 2006). High concentrations of inflammatory mediators, such as TNFα and interleukins (IL)-6 and IL-8, are commonly found in peripheral blood of COPD patients (Franciosi et al., 2006;

Higashimoto et al., 2008). A common guanine (G) to adenine (A) substitution at position 308 in the TNFα gene promoter (rs1800629) increases gene transcriptional activity (Wilson et al., 1997; Wu and McClain, 1997). This polymorphism has been studied with respect to COPD in several populations, again producing contradictory results (Higham et al., 2000; Sakao et al., 2001; Seifart et al., 2005; Brøgger et al., 2006; Chappell et al., 2007).

In the present study, we detected *CYP1A1 Msp*I and TNFα-308 polymorphisms in COPD patients, and explored the association between the polymorphisms, smoking, and COPD susceptibility in Inner Mongolia.

MATERIAL AND METHODS

Subjects

One hundred and one COPD patients and 80 control subjects were recruited from inpatients and outpatients of the Department of Respiratory of the Affiliated Hospital of Inner Mongolia Medical University and Huhhot First Hospital from 2008 to 2010. COPD was diagnosed according to the GOLD criteria: post-bronchodilator FEV1/FVC ratio <70% without significant reversibility in the presence of respiratory symptoms. Control subjects underwent routine examinations, including spirometry and chest radiography or chest computed tomography (CT). Subjects diagnosed with chronic diseases (i.e., hypertension, diabetes, cancer) or other respiratory disease (i.e., bronchial asthma, bronchiectasis, tuberculosis) were excluded from the study. The following data were recorded for each subject: name, age, gender, occupation, smoking status, history of familial diseases, and exposure to carcinogens. All subjects gave informed consent. Our study was approved by the Inner Mongolia Medical University Affiliated Hospital Ethics Committee.

DNA extraction and polymerase chain reaction-restricted fragment length polymorphism (PCR-RFLP) analysis

Two milliliter peripheral venous blood was drawn from overnight fasted subjects into sodium citrate solution, and then stored at -80°C. Genomic DNA was extracted with the BloodGen Mini Kit (CWBIO, Beijing, China). The polymorphisms *CYP1A1 Msp*I and TNFα-308 were analyzed using PCR-RFLP. PCR was performed in a 25 μL volume including 8 μL ddH_2O, 12.5 μL 2X Taq MasterMix (CWBIO), 0.25 μL 10 μM *CYP1A1* primer (sense 5'-CAG TGAAGAGGTGTAGCCGC-3' and antisense 5'-TAGGAGTCTTGTCTCATGCC-3') or TNFα primer (sense 5'-AGGCAATAGG TTTTGAGGGCCAT-3' and antisense 5'-GAGCGTCTGCTGGCTGGGTG-3'), and 4 μL template DNA. The reaction conditions were as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles of degeneration at 94°C for 30 s, reannealing at 61°C (TNFα primer at 62°C) for 30 s, and extension at 72°C for 45 s; with a final extension at 72°C for 7 min. PCR products were digested with *Msp*I and *Nco*I (Fermentas, USA) for *CYP1A1* and TNFα, respectively, in a 37°C water bath for 4 h. Ten-microliter digestion products were electrophoresed on 4% agarose gel at 80 V for 30 min and analyzed with a Gel-Pro imaging instrument.

The DNA fragment of the CYP1A1 gene was 340 bp after PCR amplification with the primer. The expected sizes of the products after digestion with *Msp*I were the following: homozygous wild-type (m1/m1) without a restriction site and an electrophoresis band of 340 bp; mutant homozygote (m2/m2) with a restriction site in each DNA chain, resulting in elec-

trophoresis bands of 200 and 140 bp; and heterozygote (m1/m2) with restriction sites in one of the DNA chains, resulting in three electrophoresis bands at 340, 200, and 140 bp.

The DNA fragment of the TNFα gene was 345 bp after PCR amplification with the primer. Homozygous wild types (GG), containing restriction sites in each DNA chain, produced two fragments of 325 and 20 bp after being digested with *Nco*I. Homozygotic mutants (AA) without a restriction site produced only one fragment of 345 bp. Heterozygotes (GA) containing restriction sites in one of the DNA chains produced three fragments of 345, 325, and 20 bp.

Statistical analysis

Statistical analysis was performed with the SPSS13.0 software. The chi-squared test or the Student *t*-test was performed to compare demographics and clinical characteristics between COPD patients and controls. The chi-squared test or Fisher's exact test were used to compare frequencies of genotypes and alleles between each group, and the chi-squared test was used to identify whether individual variants deviated from Hardy-Weinberg equilibrium. Logistic regression was used to investigate correlations among smoking, *CYP1A1 Msp*I, and the TNFα-308 polymorphism with COPD. Statistical significance was established at $P < 0.05$.

RESULTS

Clinical characteristics of COPD patients and controls

Demographics, lung function, and smoking status of COPD patients and controls are presented in Table 1. COPD patients and controls showed no difference in age or gender ($P = 0.190$ and $P = 0.191$, respectively). As expected, COPD patients had significantly limited airflow compared to controls ($P < 0.001$). There were more smokers among COPD patients than among controls ($P = 0.026$). We divided subjects into four groups according to smoking index (<1, 1-400, 400-800, >800); COPD patients and controls showed no difference in smoking index ($P = 0.173$). Compared to non-smokers, smokers had a greater risk in developing COPD [odds ratio (OR) = 2.198, 95% confidence interval (CI): 1.138-4.245, $P = 0.019$].

Table 1. Demographics, lung function, and smoking status between controls and COPD patients.

	Controls (N = 80)	COPD (N = 101)	P
Age (years)	69.50 ± 6.43	68.24 ± 6.34	0.190
Gender (M/F)	54/26	77/24	0.192
FEV1 (L)	2.18 (1.29)	0.72 (0.46)	<0.001
FVC (L)	2.62 (1.49)	1.33 (0.77)	<0.001
FEV1/FVC ratio	81.88 (5.74)	46.79 (13.74)	<0.001
Smoking	46 (57.50)	74 (73.27)	0.026
Smoking index			
<1	34 (42.50)	27 (26.73)	0.173
1-400	5 (6.25)	9 (8.91)	
401-800	16 (20.00)	27 (26.73)	
>800	25 (31.25)	38 (37.63)	

Data are reported as means ± standard deviation, median (quartile range), or number (percentage). M/F = male/female; FEV1 = forced expiratory volume in the first second; FVC = forced vital capacity; OR = odds ratio; 95%CI = 95% confidence interval.

Distribution of the *CYP1A1 Msp*I and TNFα-308 polymorphisms in COPD patients and controls

We observed that the *CYP1A1 Msp*I and TNFα-308 genotype distributions were in accordance with Hardy-Weinberg expectations in subjects (P > 0.05). This demonstrated that the study groups were representative of the population.

The genotype and allele frequencies of the *CYP1A1 Msp*I and TNFα-308 polymorphisms are shown in Table 2. The *CYP1A1 Msp*I genotypes m1/m1, m1/m2, and m2/m2 comprised 27.72, 48.51, and 23.76% of the COPD patients and 43.75, 38.75, and 17.50% of the controls, respectively (P = 0.078). The m1 and m2 allele frequencies were 51.98 and 48.02% in COPD patients and 63.13 and 36.87% in controls, respectively (P = 0.033). COPD susceptibility was higher in subjects with the m2 allele compared to subjects with the m1 allele (OR = 2.531, 95%CI = 1.297-4.940, P = 0.006).

Table 2. Distribution of genotype and allele frequencies of CYP1A1 MSPI and TNFα 308 between controls and COPD patients.

	Controls (N = 80)	COPD (N = 101)	P
CYP1A1 MSPI			
Genotype			
m1/m1	35 (43.75)	28 (27.72)	
m1/m2	31 (38.75)	49 (48.51)	
m2/m2	14 (17.50)	24 (23.76)	0.078
Allele			
m1	101 (63.13)	105 (51.98)	
m2	59 (36.87)	97 (48.02)	0.033
TNFα308			
Genotype			
G/G	71 (88.75)	73 (72.28)	
G/A	9 (11.25)	25 (24.75)	
A/A	0 (0)	3 (2.97)	0.006†
Allele			
G	151 (94.38)	171 (84.65)	
A	9 (5.62)	31 (15.35)	0.003

Data are reported as number (percentage). †Genotype G/A+A/A compared to G/G.

The TNFα-308 GG, GA, and AA genotype frequencies were 72.28, 24.75, and 2.97% in COPD patients, and were 88.75, 11.25, and 0% in controls, respectively (P = 0.006). The G and A allele frequencies were 84.65 and 15.35% in COPD patients and 94.38 and 5.62% in controls, respectively (P = 0.003). Compared to subjects with the GG genotype, subjects with GA+AA genotypes had higher COPD risk (OR = 3.639, 95%CI = 1.576-8.403, P = 0.002).

Previous studies have demonstrated that smoking is a risk factor of COPD. We investigated the association of the *CYP1A1 Msp*I and TNFα-308 polymorphisms with COPD between smoking and non-smoking subjects. There was no difference in the *CYP1A1 Msp*I polymorphism between smoking and non-smoking COPD patients and controls (P = 0.329 for genotypes and P = 0.134 for alleles; Table 3). The TNFα-308 polymorphism genotypes showed a marginally significant difference between smoking and non-smoking COPD patients and controls (P = 0.047), and allele distributions were significantly different (P = 0.030; Table 3).

Table 3. Distribution of genotype and allele frequencies of CYP1A1 MSPI and TNFα 308 between non-smoking, smoking controls, and COPD patients.

	Non-smoking controls (N = 34)	Smoking controls (N = 46)	Non-smoking COPD (N = 27)	Smoking COPD (N = 74)	P
CYP1A1 MSPI					
Genotype					
m1/m1	15 (44.12)	20 (43.48)	5 (18.52)	23 (31.08)	
m1/m2	12 (35.29)	19 (41.30)	15 (55.55)	34 (45.95)	
m2/m2	7 (20.59)	7 (15.22)	7 (25.93)	17 (22.97)	0.329
Allele					
m1	42 (61.76)	59 (64.13)	25 (46.30)	80 (54.05)	
m2	26 (38.24)	33 (35.87)	29 (53.70)	68 (45.95)	0.134
TNFα 308					
Genotype					
G/G	29 (85.29)	42 (91.30)	20 (74.07)	53 (71.62)	
G/A	5 (14.71)	4 (8.70)	6 (22.22)	19 (25.68)	
A/A	0 (0)	0 (0)	1 (3.71)	2 (2.70)	0.047†
Allele					
G	63 (92.65)	88 (95.65)	46 (85.19)	125 (84.46)	
A	5 (7.35)	4 (4.35)	8 (14.81)	23 (15.54)	0.030

Data are reported as number (percentage). †Genotype G/A+A/A compared to G/G.

DISCUSSION

COPD is characterized by local and systemic oxidative stress and chronic inflammation. The sources of oxidative stress originate from inhaled oxidants such as cigarette smoke and air pollution, and from reactive oxygen species generated by inflammatory cells. Increasing evidence suggests that genetic factors may also play a role in the pathogenesis of COPD, particularly antioxidant genes. CYP1A1 belongs to the CYP450 family and participants in the metabolism of several procarcinogens. Associations between the *CYP1A1 Msp*I polymorphism and several types of lung diseases, including lung cancer and COPD, have been investigated, but with controversial results. An association between lung cancer and the *CYP1A1 Msp*I polymorphism was found in a Japanese population (Kawajiri et al., 1990; Nakachi et al., 1991), but not in Norwegian (Tefre et al., 1991), Finnish (Hirvonen et al., 1992), or American (Shields et al., 1993) populations. This may be related to the relatively lower m2 allele frequency observed in Caucasian control subjects (approximately 14%) compared to a frequency of 33% in the Japanese population. The m2 allele frequency in the controls of the present study was 36.87%, which is similar to that of the Japanese population and higher than that of Caucasian populations. Studies investigating the relationship between the *CYP1A1 Msp*I polymorphism and COPD are scarce. Our results found no difference in genotype frequencies of the *CYP1A1 Msp*I polymorphism between COPD patients and controls (P = 0.078), and similar results were reported in a study in a Taiwanese population (Cheng et al., 2009). In the present study, COPD susceptibility was found to be higher in subjects carrying the m2 allele compared to subjects with the m1 allele (OR = 2.531, 95%CI = 1.297-4.940, P = 0.006). To confirm our results, the study should be repeated with larger samples in the future.

Chronic inflammation is also a key factor that contributes to COPD. Increased levels of inflammatory proteins, such as IL-6, C-reactive protein, and TNFα, have been linked to COPD (Gan et al., 2004; Garcia-Rio et al., 2010; Pelegrino et al., 2013). Studies have shown that TNFα plays roles in weight loss and muscle reduction in COPD patients (Takabatake et al., 2000; Eid et al., 2001; Higashimoto et al., 2008). Increased TNFα is secreted from active

macrophages through chronic inflammation. Furthermore, a single nucleotide polymorphism at position 308 of the TNFα gene promoter results in elevated gene expression. In the present study, TNFα-308 genotype and allele frequencies were significantly different between COPD patients and controls (P = 0.006 and P = 0.003, respectively). Moreover, there was a difference in the TNFα-308 polymorphism between smoking and non-smoking COPD patients and controls (P = 0.047 for genotypes and P = 0.030 for alleles), indicating that the TNFα-308 polymorphism might interact with tobacco consumption. Smoking status has previously been shown to have a potential influence on TNFα levels (Tanni et al., 2010). Studies in Taiwanese (Huang et al., 1997), Japanese (Sakao et al., 2001), and Caucasian smokers (Gingo et al., 2008) showed similar results to those of the present study in demonstrating that the TNFα-308 polymorphism is a risk factor of COPD. However, studies in Thai (Chierakul et al., 2005), adenovirus C-infected Egyptian (Ezzeldin et al., 2012), Caucasian (Higham et al., 2000; Chappell et al., 2007), German (Seifart et al., 2005), and European-descended American (Tanaka et al., 2007) subjects showed opposite results. Teramoto et al. (2008) stated that the TNFα-308 polymorphism was not associated with susceptibility to Asian COPD. Recently, two meta-analyses found that the association of the TNFα-308 polymorphism and COPD was statistically significant in Asian but not in Caucasian populations, for both the entire and only the smoking population (Zhan et al., 2011; Zhang et al., 2011). Another meta-analysis also confirmed that there was no association between the TNFα-308 polymorphism and COPD in Caucasian populations (Brøgger et al., 2006). The two more recent meta-analyses were based on more studies than Teramoto et al. (2008), and in particular, included studies from the Chinese National Knowledge Infrastructure (CNKI) database, which might explain their controversial conclusions about the TNFα-308 polymorphism and COPD in Asians.

In summary, subjects with the *CYP1A1 Msp*I m2 allele or the TNFα-308 A allele had higher COPD susceptibility (OR = 2.531, 95%CI = 1.297-4.940, P = 0.006 and OR = 3.639, 95%CI = 1.576-8.403, P = 0.002, respectively). However, only the TNFα-308 polymorphism, and not the *CYP1A1 Msp*I polymorphism, might exert an interaction with smoking.

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