

Full-length article

Transforming growth factor- β 1 gene polymorphisms associated with chronic obstructive pulmonary disease in Chinese population¹Zhi-guang SU², Fu-qiang WEN, Yu-lin FENG, Min XIAO, Xiao-ling WU*Laboratory of Pulmonary Medicine, West China Hospital, Sichuan University; Key Laboratory of Biotherapy of Human Disease, Ministry of Education, Chengdu 610041, China***Key words**chronic obstructive pulmonary disease; transforming growth factor- β 1; single nucleotide polymorphisms; haplotypes¹ Project supported by the National Natural Science Foundation of China (No 30200161 and No 30370628).² Correspondence to Dr Zhi-guang SU.

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Abstract

Aim: To determine the frequencies of polymorphism and haplotype in the transforming growth factor-beta 1 (TGF- β 1) gene promoter in the Chinese population and to investigate the susceptibility of this population to chronic obstructive pulmonary disease (COPD). **Methods:** The target fragments of the TGF- β 1 gene promoter were amplified and analyzed by polymerase chain reaction-restriction fragment length polymorphism technique in 84 COPD patients and 97 age- and sex-matched healthy controls. The test for Hardy-Weinberg equilibrium was performed using HWE program of the LINKUTIL package and statistical analysis was carried out with the SPSS statistical package. An expectation maximization algorithm was used for the pairwise linkage disequilibrium test and haplotype analysis. **Results:** More carriers of the -800A allele, or fewer carriers of the -509T allele, were detected in the COPD patients compared with the non-symptomatic control subjects [for the -800A allele, 29.8% vs 14.4%, respectively, $\chi^2=6.257$, degrees of freedom (df)=1, $P=0.012$; for the -509T allele, 27.3% vs 44.3%, respectively, $\chi^2=5.582$, df=1, $P=0.018$]. The prevalence of the -800A allele was significantly higher in the COPD patients than in control subjects ($P=0.009$), whereas the frequency of the -509T allele was significantly higher in control subjects than in the COPD patients ($P=0.008$). In addition, this distribution tendency for the -800A or -509T allele was similar in heavy smokers (smoking history ≥ 20 pack years); (number of packs of cigarettes per day multiplied by the number of years of smoking) $\chi^2=7.235$, $P=0.007$, and $\chi^2=5.636$, $P=0.018$, respectively). The linkage disequilibrium was found between -800 G \rightarrow A and -509 C \rightarrow T ($D>0.60$, $P<0.0001$), and the frequency of the AC haplotype, consisting of the least common base at -800 and the most common base at -509, was significantly higher in patients with COPD than in controls (0.056 vs 0.021, $P<0.05$). **Conclusions:** The single nucleotide polymorphism (SNP) in the TGF- β 1 gene promoter might be associated with COPD, and the -800A/-509C haplotype is possibly one of the susceptibility factors for COPD.

Introduction

Chronic obstructive pulmonary disease (COPD) is currently the leading cause of disability-adjusted life years. Globally, it is projected to be the 3rd most important cause of death by the year 2020, and it is estimated that the national prevalence is up to 3.1% in the Chinese adult population.

Cigarette smoking is the major risk factor for COPD; however, only approximately 15% of smokers develop clinically relevant airflow obstruction^[1]. This variation in the susceptibility to cigarette smoke in combination with the familiar aggregation of COPD suggests that there may be a genetic component to the development of COPD. Multiple studies in diverse populations have shown evidence for a large

genetic contribution to the variability in pulmonary function and for the familial aggregation of COPD patients^[2,3]. As expected, segregation analysis suggests that multiple genes may be involved. At present, however, only a single gene, α 1-antitrypsin, a potent inhibitor of inflammatory cell protease in the lung, has been unequivocally implicated in the development of COPD. The association between the ZZ type polymorphism of this gene and COPD has been established^[4]. Furthermore, the associations between COPD and polymorphisms in several other genes of potential importance to COPD pathogenesis also have been studied. These include α 1-antichymotrypsin^[5], microsomal epoxide hydrolase^[6], vitamin D-binding protein^[7], and tumor necrosis factor- α ^[8].

Transforming growth factor- β (TGF- β) is a multifunctional cytokine that regulates the proliferation and differentiation of a wide variety of cell types *in vitro*. In humans, the TGF- β family includes 3 isoforms (TGF- β 1, TGF- β 2, and TGF- β 3) with great structural and functional similarities^[9]. TGF- β 1 is the most abundant isoform and is highly conserved in primary sequence through evolution. It is synthesized as precursor latent forms (390 amino acids long), which have no known biological activity^[10]. The active form of TGF- β 1 is released proteolytically from the precursor form^[11] and consists of a disulphide-linked 25 kDa homodimer of two 112 amino acid peptides. The coding region of the TGF- β 1 gene consists of 7 exons and 6 introns^[12].

The expression of TGF- β 1 is influenced by polymorphisms in the TGF- β 1 gene, and some of these polymorphisms may be associated with COPD and other diseases^[13-15]. Wu *et al* investigated the association between the single nucleotide polymorphism T+869C (Leu10Pro) at exon 1 of the TGF- β 1 gene with COPD in a Caucasian Americans white population^[16]. There are several registered polymorphisms within the TGF- β 1 gene that might be functional and might be associated with COPD. In particular, a promoter polymorphism at -509 C/T is associated with the diagnosis of asthma and may enhance TGF- β 1 gene transcription^[17], but whether there is an association between the -509 C/T polymorphism and COPD has not yet been investigated. The aim of present study was to screen for DNA sequence variants in the TGF- β 1 promoter, to establish the prevalence of each genotype in individuals with COPD and controls in a Chinese population, and to identify any haplotypes associated with a predisposition to COPD.

Materials and methods

Subjects Eighty-four patients with COPD were recruited

from the West China Hospital, Sichuan University, Chengdu, China. The definition of COPD was consistent with that in the American Thoracic Society (ATS) consensus statement. The patients had a history of chronic or recurrent productive cough for >2 years and decreased maximum expiratory flow, which had been slowly progressive and irreversible. The presence of other lung or cardiac diseases as the cause of patient symptoms was excluded by clinical and radiographic examinations. The criteria for enrolment were as follows: (i) individuals with a forced expiratory volume in 1 s (FEV₁) < 70% of predicted, an FEV₁/forced vital capacity (FVC) ratio of < 70%, and an increase in FEV₁ of < 12% 15 min after the inhalation of 400 μ g Fenoterol HBr MDI (Berotec; Boehringer Ingelheim, Ridgefield, CT, USA); and (ii) patient consent to participate in the study. Most of the patients were receiving oral methylxanthine, inhaled anticholinergic agents, and inhaled β 2-agonists as needed. The patient's name, age, sex, family history, smoking habits, the number of cigarettes smoked, the duration of diseases, and chest radiographic findings were recorded. Pulmonary function testing (CHESTAC-33-8800, Chest Ltd, Tokyo, Japan) was performed according to the ATS performance requirements. Ninety-seven unrelated, age-matched healthy subjects, who had no known medical illness or family disorders and were not taking any medications, acted as control subjects. This study was approved by the Internal Review Board of the West China Hospital, Sichuan University, and signed informed consent forms were obtained from all subjects.

DNA preparation and polymerase chain reaction amplification Genomic DNA was prepared from peripheral blood leukocytes using the "salting-out"^[18] procedure and stored at 4 °C. Fragments containing the single nucleotide polymorphism (SNP) G-800A or T-509C were amplified by polymerase chain reaction (PCR). The primer sequence was based on the DNA sequence from GenBank accession numbers NM_000660 and X05839. For the detection of polymorphism at nucleotide acid -800, the primer pairs were 5'-CTGGCAGT-TGGCGAGAACA-3' (sense) and 5'-TAGAAAGGACAGAA-GCGGTG-3' (antisense); and the generated PCR product size was 326 base pairs (bp). The primer pairs for delineating the polymorphism at nucleotide acid -509 were 5'-CCAGCTAAGGCATGGC ACCG-3' (sense) and 5'-GCGGTGTGGTCA-CCAGAGA-3' (antisense); the PCR product size from these primers was 300 bp. Each PCR amplification mixture contained 0.1 μ g genomic DNA, 0.2 μ mol/L primers, 50 μ mol/L deoxyribonucleotide triphosphate (dNTP), and standard PCR buffer in a total volume of 50 μ L. The reaction mixture was preheated at 94 °C for 4 min. Subsequently, 0.4 units of *Taq* polymerase was added. The 30 cycles of PCR amplification

were performed with a temperature profile consisting of denaturation at 94 °C for 45 s, annealing at 58 °C and 61 °C for 30 s, extension at 72 °C for 30 s, and final extension at 72 °C for 10 min. The reactions were carried out in a PTC-200 Programmable Thermal Controller (MJ Research, Watertown, MA, USA).

Genotyping Genotyping for the -800G/A and -509C/T polymorphisms was performed by digestion of PCR product with the restriction enzymes MaeIII and SauI (Amersham Biosciences, Piscataway, NJ, USA), respectively. After PCR, 10 µL of the reaction mixture was digested with 1 U MaeIII or SauI in 1×buffer M (Amersham Biosciences) for 3 h at 37 °C. The digest mixture was resolved on a 2.0% agarose gel stained with ethidium bromide. For the -800G/A polymorphism, DNA from individuals with the homozygous G genotype (GG) produced 1 band at 326 bp; the homozygous A genotype (AA) produced 2 bands, 1 at 206 bp and 1 at 120 bp; and the heterozygous genotype (GA) produced all 3 bands. A gel of the restriction fragment length polymorphism digestion products is shown in Figure 1A. For the -509C/T polymorphism, individuals with the homozygous C genotype (CC) produced 1 band at 300 bp; the homozygous T genotype (TT) produced 2 bands, 1 at 202 bp and 1 at 98 bp; and the heterozygous genotype (CT) produced all 3 bands, as shown in Figure 1B. Control DNA samples representative of each of the different genotypes were included in each digestion. Repeat genotyping was performed on 5 of every 100 samples chosen by random selection. Genotyping errors are estimated to have occurred at a frequency of <1%.

Statistical analysis Data analyses were performed with the Statistical Package for the Social Science (SPSS, version 10.0, Inc in Chicago, Illinois, USA). Data were expressed as mean±SEM. The significance level for statistical tests was taken to be 0.05. Deviation of the genotype counts from the

Hardy-Weinberg equilibrium was tested using a χ^2 test with 1 degree of freedom (df). Differences between the patients with COPD and the controls with respect to the allele frequencies and genotype distributions were analyzed by χ^2 test or Fisher's exact test when necessary. Haplotype frequencies for pairs of alleles, as well as χ^2 values for allele associations, were estimated by the Estimating Haplotype-frequencies software program^[19-20]. Linkage disequilibrium coefficients $D'=D/D_{\max}$ (D is the difference between the observed and the expected gamete frequency, D_{\max} is the maximum disequilibrium, which occurs when all double heterozygotes are either in linkage phase (AB/ab) or in repulsions phase (Ab/aB). were calculated by the 2LD program^[21].

Results

General characteristics The study population consisted of 84 patients with COPD and 97 control subjects (Table 1).

Table 1. General characteristics of the study population (mean±SEM). FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity.

| Variable | Controls (n=97) | COPD (n=84) | P |
|-------------------------------|-----------------|-------------|-------|
| Age/years | 65±8 | 63±9 | >0.1 |
| No. men/% | 83 (85.6) | 74 (88.1) | >0.1 |
| Smoking history | | | |
| 0–20 pack years ¹ | 26 | 17 | >0.05 |
| ≥20 pack years | 71 | 67 | >0.05 |
| FEV ₁ | 1.87±0.60 | 0.97±0.32 | <0.01 |
| FEV ₁ /Predicted/% | 93.7±3.4 | 49.0±0.4 | <0.01 |
| FEV ₁ /FVC | 78.0±4.6 | 59.2±8.3 | <0.01 |

¹ Pack years: number of packs of cigarettes per day multiplied by the number of years of smoking.

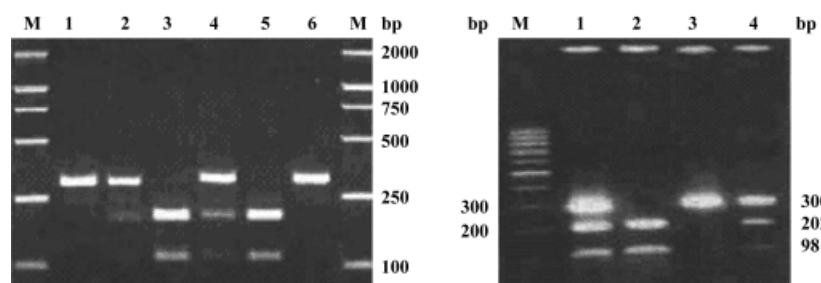


Figure 1. Agarose gel showing restriction fragment length polymorphism for polymorphisms in the transforming growth factor- β 1 gene at nucleotide -800 (A) and nucleotide -509 (B). (A) M: marker, 250 bp DNA ladder; lanes 1 and 6 show the GG genotype; lanes 2 and 4 show the GA genotype, and lanes 3 and 5 show the AA genotype. (B) M, 100 bp DNA ladder; lane 2 shows the TT genotype; lane 3 shows the CC genotype; and lanes 1 and 4 show the TC genotype.

The COPD cases and control subjects did not differ significantly in sex, age, and smoking history characteristics. The parameters used for FEV_1 , FEV_1 /predicted, and FEV_1 /FVC were significantly decreased in the COPD case subjects compared with the controls (Table 1, $P < 0.01$).

Distribution of the G-800A and C-509T in COPD patients and controls To determine the prevalence of the SNP G-800A and C-509T, we screened them in all the 84 COPD patients and in the 97 controls. The genotype distribution and allele frequencies are listed in Table 2. No deviation from Hardy-Weinberg equilibrium (for SNP G-800A, $\chi^2 = 1.379$, $df = 1$, $P = 0.240$ in the COPD group, $\chi^2 = 1.737$, $df = 1$, $P = 0.188$ in controls; for SNP C-509T, $\chi^2 = 1.488$, $df = 1$, $P = 0.222$ in the COPD group, $\chi^2 = 2.400$, $df = 1$, $P = 0.121$ in controls) was noted in the COPD or control groups. As a result, more carriers of the -800A allele, or fewer carriers of -509T allele, were detected in the COPD patients compared with in the control subjects (for the -800A allele, 29.8% vs 14.4%, $\chi^2 = 6.257$, $df = 1$, $P = 0.012$; for the -509T allele, 27.3% vs 44.3%, $\chi^2 = 5.582$, $df = 1$, $P = 0.018$). The prevalence of the -800A allele was significantly higher in COPD patients than in control subjects ($P = 0.009$), whereas the frequency of -509T allele was significantly decreased in COPD patients compared with

that in control subjects ($P = 0.008$).

Relationship between cigarette smoking and the distribution of TGF-β1 gene polymorphisms The allele and genotype distributions were compared in heavy smokers (smoking history ≥ 20 pack years (number of packs of cigarettes per day multiplied by the number of years of smoking) to determine whether the prevalence of different alleles or genotypes was associated with smoking. Results showed that the frequency of the -800A allele was still significantly higher and the frequency of the -509T allele was significantly decreased in patients with COPD (group A) than in control subjects (group B) ($\chi^2 = 7.235$, $P = 0.007$, and $\chi^2 = 5.636$, $P = 0.018$, respectively; Table 3).

Linkage disequilibrium between -800G/A and -509C-T polymorphisms in the TGF-β1 gene We analyzed the relationship between -800G/A and -509C-T polymorphisms and their effects on COPD. The extent of D in pairwise combinations of alleles in loci at the TGF-β1 promoter was estimated by means of maximum likelihood from the frequency of diploid genotypes in the patients with asthma and controls. Haplotype frequencies and the coefficient of linkage disequilibrium (D') are given in Table 4. It was clear that the D' values for polymorphisms at nucleotides -800 bp and -509 bp

Table 2. Frequency distributions of the transforming growth factor-β1 gene in patients with chronic obstructive pulmonary disease versus controls.

| SNP ¹ | Genotype | Control n (%) | Case n (%) | χ^2 | P | Allele | Control n (%) | Case n (%) | χ^2 | P |
|------------------|----------|------------------|---------------|----------|-------|--------|------------------|---------------|----------|-------|
| -800 G/A | GG | 83 (85.6) | 59 (70.2) | 6.47 | 0.039 | GA | 179 (92.3) | 140 (83.3) | 6.866 | 0.009 |
| | GA | 13 (13.4) | 22 (26.2) | | | | 15 (7.7) | 28 (16.7) | | |
| | AA | 1 (1) | 3 (3.6) | | | | | | | |
| -509 C/T | CC | 54 (55.7) | 61 (72.6) | 6.627 | 0.036 | CT | 143 (73.7) | 143 (85.1) | 7.064 | 0.008 |
| | CT | 35 (36.1) | 21 (25.0) | | | | 51 (26.3) | 25 (14.9) | | |
| | TT | 8 (8.2) | 2 (2.4) | | | | | | | |

¹SNP: single nucleotide polymorphism age (years).

Table 3. Distribution of the alleles and genotypes of the transforming growth factor-β1 gene in smokers with or without chronic obstructive pulmonary disease.

| Group | n | -800 G/A | | | | | -509 C/T | | | | |
|-------|----|----------------|-----------|---------|--------------|-----------|----------------|-----------|---------|--------------|-----------|
| | | Genotypes (n%) | | | Alleles (n%) | | Genotypes (n%) | | | Alleles (n%) | |
| | | GG | GA | AA | G | A | CC | CT | TT | C | T |
| A | 67 | 44 (65.7) | 21 (31.3) | 2 (3.0) | 109 (81.3) | 25 (18.7) | 47 (70.1) | 19 (28.4) | 1 (1.5) | 103 (72.5) | 21 (15.7) |
| B | 71 | 60 (84.5) | 11 (15.5) | 0 (0.0) | 131 (92.3) | 11 (7.7) | 37 (52.1) | 29 (40.8) | 5 (7.1) | 113 (84.3) | 39 (27.5) |

Table 4. Estimate of pairwise haplotype frequencies and disequilibrium statistics.

| Polymorphic sites and subjects | Estimated haplotype frequency | | | | D (difference between observed and expected gamete frequency) | D_{\max} (maximum disequilibrium) | D' (linkage disequilibrium coefficients) | P |
|--------------------------------|-------------------------------|-------|-------|-------|---|-------------------------------------|--|---------|
| | GC | AC | GT | AT | | | | |
| G-800A/C-509T | | | | | | | | |
| COPD patients | 0.795 | 0.056 | 0.088 | 0.061 | 0.086 | 0.124 | 0.691 | <0.0001 |
| Controls | 0.716 | 0.021 | 0.207 | 0.056 | 0.036 | 0.057 | 0.632 | <0.0001 |

differ significantly from zero, and the frequency of the AC haplotype, consisting of the least common base at -800 and the most common base at -509, was significantly higher in patients with COPD than in controls (0.056 vs 0.021, respectively, $P < 0.05$).

Discussion

We have shown that the A allele at position -800 in the promoter region of the TGF- β 1 gene is more common and the T allele at position -509 is less common in subjects with COPD than in control subjects. Some evidence has shown a correlation between the 2 polymorphisms (G-800A, C-509T) and the concentration of TGF- β 1 in human plasma^[22]. The G-800A substitution is thought to disrupt a consensus half-site for the binding of the nuclear transcription factor CRE (cyclic AMP-responsive element)-binding protein, and it was found that the presence of the A allele is significantly associated ($P < 0.05$) with lower levels of total TGF- β 1 in the circulation^[22]. Conversely, the -509T allele has been shown to be associated with higher levels of TGF- β 1 in serum and increased TGF- β 1 mRNA in peripheral blood mononuclear cells^[22]. These findings indicate that the G-800A and C-509T polymorphisms may be involved in the modulation of expression of the TGF- β 1 gene and therefore a predisposition for COPD could be linked to particular alleles of this gene. The high producer genotype for TGF- β 1 may protect against the development of COPD. A recent study by Wu *et al* has shown that the +869Pro allele, associated with higher serum concentrations of TGF- β 1, was less common in subjects with COPD than in control subjects^[16].

A recent study using an animal model of emphysema adds plausibility to the suggestion that increased production of TGF- β 1 can protect against the development of COPD. The integrin $\alpha_v\beta_6$ activates latent TGF- β ^[23]. Mice that lack this integrin (Itgb6 null mice) develop age-related emphysema. When transgenes for TGF- β 1 were inserted into these mice

so that they constitutively expressed active TGF- β 1, the changes in the lung associated with the deletion of the integrin gene no longer occurred. In these mice, the productive effects of TGF- β 1 appear to be related to inhibition of macrophage metalloelastase (MMP12), which degrades elastin.

More than 100 SNP and other genetic variants have been identified in genes of the TGF- β 1 signaling pathway, and a few of these have been associated with disease^[13]. C-509T was chosen as the sole candidate SNP because previous studies suggest that it is associated with altered serum levels of TGF- β 1, asthma diagnosis, asthma severity, and serum IgE levels^[22,24,25]. For example, in a study involving 84 monozygous and 86 dizygous twins, Grainger *et al* showed that TGF- β 1 levels in plasma were under genetic control (heritability estimate 0.54) with the C-509T SNP being responsible for 8.2% of the additive genetic variance^[22]. The T allele was associated with higher levels of TGF- β 1 than the C allele was, and there was an allele dose effect, with the highest levels in TT individuals (7.62 ng/mL), intermediate levels in CT individuals (5.06 ng/mL), and the lowest levels in CC individuals (3.83 ng/mL)^[25].

A wide variety of functions have been attributed to TGF- β 1 (such as reduction of inflammation and promotion of wound healing^[26], immunosuppression^[27], regulation of cell proliferation, differentiation and migration^[9]), and regulation of extracellular matrix production^[28,29]. Some of these functions could protect against the development of COPD. TGF- β 1 can inhibit matrix metalloproteinases that may contribute to the development of emphysema through the digestion of elastic fibers^[30,31]. It also promotes the formation of elastin^[32,33] and this could help repair damage to the lungs of individuals who are smokers and who are risk of developing COPD. Recently, TGF- β 1 was located on chromosome 19q, a genomic region linked to the diagnosis of COPD in some genome-wide scans^[34]. Our study strengthens this association between TGF- β 1 and COPD.

In summary, our results suggest that genetic variation at

the promoter of the TGF- β 1 gene might be associated with TGF- β 1 levels and predisposition to the complex COPD. The distributions of TGF- β 1 polymorphisms are different between different ethnic groups. Further replication is essential for the validation of this association in Caucasian, African, or other ethnic populations, and, to elucidate the role of TGF- β 1 in COPD, future work is needed to establish the exact effect of TGF- β 1 polymorphisms on the activation process of the protein and its function.

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