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Transforming Growth Factor-β1 and Tumor Necrosis Factor-α are Associated with Clinical Severity and Airflow Limitation of COPD in an Additive Manner

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Abstract
Background The role of tumor necrosis factor-α (TNF-α) and transforming growth factor-β1 (TGF-β1) in chronic obstructive pulmonary disease (COPD) is controversial. The purpose of this study was to assess the relationships among polymorphisms, clinical phenotypes, and the serum levels of TNF-α and TGF-β1.
Methods Polymorphisms of promoters of TNF-α (rs 361525 and rs 1800629) and TGF-β1 (rs 1800469) in 110 COPD patients, 110 nonsmoker health controls without COPD, and 34 smokers were evaluated. Pulmonary functions, chest computed tomography, TGF-β1, and TNF-α were assessed.
Results The genetic polymorphism of TNF-α (rs 361525) was associated with COPD. More severe COPD patients had higher serum levels of TNF-α and TGF-β1; moreover, serum levels of TGF-β1of mild COPD patients were higher than normal controls. All of the studied subjects were divided into four groups by the 95th percentile value of control as cutoff serum value of TGF-β1 (224.35 ρg/ml) or TNF-α (17.56 ρg/ml) to define the high value of TGF-β1 or TNF-α, which are higher than those cutoff of values (>224.35 or 17.56 ρg/ml). The FEV_1 of the group with high TGF-β1 + low TNF-α or low TGF-β1 + high TNF-α or high TNF-α + high TGF-β1 was lower than the group with low TGF-β1 + low TNF-α group. Moreover, the lowest value of FEV_1 was in the group with high TNF-α + high TGF-β1.
Conclusions The genetic polymorphism of the TNF-α is associated with COPD. Both TGF-β1 and TNF-α modulate clinical severity and airflow limitation in an additive manner.

Keywords Chronic obstructive pulmonary disease · Gene polymorphism · Tumor necrosis factor-α · Transforming growth factor-β1 · Phenotype

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Introduction

Chronic pulmonary obstructive pulmonary disease (COPD) is thought to be intimately linked to local lung and systemic inflammation [1–3]. As a consequence, attention has been focused on the levels of inflammatory and remodeling biomarkers, which are related to clinical and physiological measurements [4–8]. The severity of stable COPD can be difficult to assess clinically. COPD severity has been addressed using pulmonary function data to assess the airway obstruction. However, it is difficult for some patients, especially elder patients to perform pulmonary function test. Identifying useful biomarkers that correlate with clinical symptoms and airway obstruction would be a very important addition to clinical staging of stable COPD. Stable COPD patients still have chronic inflammation and remodeling in the airway as well as systemic inflammation. Therefore, we hypothesize that identification of biomarkers of inflammation and remodeling in COPD may be relevant for assessing the severity of COPD.

Tumor necrosis-α (TNF-α) has been shown to be a highly proinflammatory cytokine in COPD, as it upregulates adhesion molecules, increases mucin secretion, and promotes airway remodeling. TNF-α is produced by a large number of cells in the airways, including mast cells, smooth muscle cells, epithelial cells, monocytes, and macrophages. This cytokine has been shown to be relevant, being increased in patients with COPD [9–11].

Transforming growth factor-β1 (TGF-β1) is one of the main mediators involved in tissue remodeling in the lung. This profibrotic cytokine is produced by a number of cells, including macrophages, epithelial cells, fibroblasts, and eosinophils. Increased expression of TGF-β1 in small airways epithelium and bronchial reticular basement membranes in patients with COPD was reported [12, 13]. TGF-β1 is believed to play an important role in most of the cellular biological processes, which is leading to airway remodeling. It was shown to be involved in epithelial changes, subepithelial fibrosis, airway smooth muscle remodeling, and microvascular changes [12, 13].

To date, association between COPD and genetic polymorphisms of TNF-α or TGF-β1 remains uncertain [14–19]. Furthermore, concentration of serum with TNF-α or TGF-β1 in COPD has not been explored adequately to assess correlations with clinical phenotypes. Based on previous studies, we selected potentially relevant single-nucleotide polymorphisms of TNF-α or TGF-β1 in COPD patients and investigated the association between the genetic polymorphisms of TNF-α or TGF-β1 with COPD. Then, we studied whether serum levels of TNF-α or TGF-β1 are associated with COPD clinical severity and airflow limitation, and moreover, whether there is additive effect on COPD severity by combination of both TNF-α and TGF-β1.

Materials and Methods

The hospital review board for human studies approved the study protocol. Informed consent from each subject was obtained before participation.

Study Subjects

Controls and Stable COPD

A cohort of 110 stable COPD patients, who were diagnosed and followed in the outpatient department of Taipei Veterans General Hospital, 110 nonsmoker control subjects, and 34 smoker controls (healthy with no COPD) were recruited for this study (Table 1). COPD was diagnosed on the basis of history, chest radiography findings, physical examination, and spirometric data, which are based on the COPD diagnostic criteria of the global initiative for chronic obstructive lung disease (GOLD) criteria [2]. All COPD subjects had an FEV_1/FVC ratio <0.7 after inhaled bronchodilator. According to the GOLD classification severity, patients were stratified: 38 (35 %) stage I mild COPD: $FEV_1\ \% > 80$; 50 (45 %) stage II moderate COPD: $FEV_1\ \% \ 50 \leq FEV_1\ \% < 80$; 17 (15 %) stage III severe: $30 \leq FEV_1\ \% < 50$); 5 (5 %) stage IV very severe COPD: $FEV_1\ \% < 30$ or $FEV_1\ \% < 50$ plus respiratory failure or heart failure. The exclusion criteria included asthma, cardiovascular disease, infection, malignant disease, rheumatoid diseases, and other severe comorbidities, including who was treated with anti-inflammatory and immunomodulatory drugs. Stable COPD was defined as disease without reported exacerbations and at least three regular visits during 6 months without any change in respiratory medications and absence of infections in the study. On the basis of high-resolution computed tomography (HRCT), lung function tests and clinical manifestations, all COPD patients were classified into three phenotype groups: chronic bronchitis (chronic cough with daily sputum and airway wall thickness in HRCT), emphysema (exertion dyspnea and emphysematous change in HRCT), and mixed types.

The control group included 110 nonsmokers and 34 asymptomatic smokers without clinical or physiologic evidence of COPD. All control subjects visited the hospital for a health examination. All control subjects had normal pulmonary function ($FEV_1/FVC > 70$ % and $FEV_1 > 80$ % of the predicted value) and no comorbidities. Control subjects were matched to COPD subjects with respect to age. Peripheral blood was collected from patients with COPD and control subjects in the morning between 9 and 10 AM. The plasma was kept at −80 °C until analysis by a technician who was blinded to the condition of the patients.

Pulmonary Function Test

Pulmonary function (FVC, FEV_1, FEV_1/FVC) was assessed by automated method using body plethysmography (6200 Autobox DL, SensorMedics, Yorba Linda, CA). These measures were assessed by the guidelines of the American Thoracic Society [20].

Genotyping

DNA was extracted from blood samples with either a commercial kit (QIAamp Blood Kit; Qiagen, Chatsworth, CA) or an automated nucleic acid purification system (Genepure; Applied Biosystems, Foster City, CA).

Promoter of tumor necrosis-α (TNF-α-238 G/A locus: rs 361525 and TNF-α-308 G/A locus: rs 1800629)

The TNF-α G-238 A promoter gene was amplified by 35 cycles of PCR using sense primer 5′-ATC TGG AGG AAG

Table 1 Clinical characteristics of COPD and control groups

Groups	Control ($n = 110$)	Control smoking ($n = 34$)	COPD ($n = 110$)				*P* value
			Mild ($n = 38$)	Moderate ($n = 50$)	Severe ($n = 17$)	Very severe ($n = 5$)	
Age	72.41 ± 12.28	72.6 ± 7.34	77.2 ± 3.87	74.25 ± 5.26	72.33 ± 0.94	75.83 ± 8.19	>0.05
Pack-years	0 ± 0	36.4 ± 42.87	48.09 ± 37.71	32.57 ± 21.07	43.2 ± 28.33	96.25 ± 34.03	<0.05
FVC (Liters)	3.33 ± 0.70	3.18 ± 0.54	2.52 ± 0.66	2.62 ± 0.6	2.11 ± 0.43	2.16 ± 1.02	<0.001
FVC (%pred)	100.09 ± 12.87	99.85 ± 16.23	85.19 ± 18.78	80.53 ± 14.31	71 ± 14.17	66.33 ± 28.95	<0.05
FEV_1 (Liters)	3.03 ± 0.67	2.94 ± 0.49	2.1 ± 0.36	1.55 ± 0.39	0.94 ± 0.24	0.66 ± 0.22	<0.001
FEV_1 (%pred)	97.66 ± 10.59	94.92 ± 12.96	85.55 ± 17.00	68.23 ± 12.08	42.69 ± 5.77	26.5 ± 7.32	<0.001
FEV_1/FVC (%)	91.87 ± 10.4	79.94 ± 8.85	66.18 ± 4.92	59.46 ± 7.39	45.18 ± 7.98	39.67 ± 21.22	<0.001

Data are mean ± SD

FVC force vital capacity, FEV_1 volume of force expiration at first second, FEV_1 *(%pred)* % predict value of FEV_1

CGG TAG TG and antisense primer 5′-AGA AGA CCC CCC TCG GAA CC. PCR was performed in a Perkin-Elmer GeneAmp PCR system 9700 (Perkin-Elmer Medical Instruments, Pomona, CA). After PCR, 10 μl of the reaction mixture was digested with 1 U MspI (New England Biolabs (NBL), Beverly, MA). The digest mixture was resolved on a 3 % agarose gel stained with ethidium bromide. DNA from individuals with the homozygous G genotype (GG) produced one band at 152 bp; the homozygous A genotype (AA) produced one band at 133 bp; and the heterozygous genotype (GA) produced all two bands [21].

Tumor necrosis-α G-308A polymorphism was analyzed by PCR combined with restriction fragment length polymorphism (RFLP). Fragments were amplified in a total volume of 15 μl. The utilized primer, restriction enzyme, and expect products were as follows: The primers were 5′-AGG CAA TAG GTT TTG AGG GCC AT-3′ and 5′-TCC TCC CTG CTC CGA TTC CG-3′, and the restriction enzyme was *Nco*I (New England BioLabs); the −308G allele yielded a single 107 bp fragment, and the −308A allele yielded 87 and 20 bp fragments, respectively [22].

Promoter of Transforming Growth Factor-β1 (TGF-β1;-509C/T Locus: rs 1800469)

An amplification of 406 bp was generated by 35 cycles of PCR using sense primer 5′-CCGCTTCTGTCCTTTCTAGG and antisense primer 5′-AAAGCGGGTGATCCAGATG. PCR was performed. After PCR, 10 μl of the reaction mixture was digested with 1 U *Eco*81I (*Sau*I) (Amersham Biosciences, Piscataway, NJ). The digest mixture was resolved on a 1.5 % agarose gel stained with ethidium bromide. DNA from individuals with the homozygous C genotype (CC) produced two bands: one at 223 bp and one at 183 bp; the homozygous T genotype (TT) produced one band at 406 bp; and the heterozygous genotype (CT) produced all three bands [13].

Blood Sampling and Analysis of Serum Content of TNF-α and TGF-β1

The levels of TGF-β1 and TNF-α in serum were assayed by a standardized sandwich enzyme-linked immunosorbent assay (ELISA) method (Invitrogen Corporation, Camarillo, CA). The absorbance was read at 450 nm (SpectraMax M5, Molecular Devices, USA).

Statistical Analysis

The values for FEV_1 and serum levels of TGF-β1 and TNF-α were expressed as mean ± SD. The frequency genotypes were expressed as the number and percentage of the total. The correlation between TGF-β1 or TNF-α promoter polymorphisms of genotypes and alleles frequency in COPD, non-COPD smoker, and nonsmoker healthy subjects by the Fisher's exact test, χ^2 test or Pearson χ^2 test. The ANOVA test was used to compare the values for FEV_1, TGF-β1, or TNF-α across the three genotypes or the serum level of TGF-β1 or TNF-α in various severity or phenotypes of COPD patients.

Results

Association Between TGF-β1 or TNF-α Polymorphisms and COPD

Selected candidate genetic polymorphisms of TGF-β1 and TNF-α at promoter 308G/A was not associated with COPD (Tables 2, 3), but TNF-α 238 G/A polymorphism with G

Table 2 Association of COPD with polymorphisms of the TGF-β1 promoter

Groups	Genotypes			Allele frequency		*P1* value	OR1	95 % CI1	*P2* value	OR2	95 % CI2
	CC	CT	TT	C	T						
Normal ($n = 110$)	8 (7.3 %)	82 (74.5 %)	20 (18.2 %)	0.45	0.55	0.659	1.0432	0.8629–1.2611			
Smoker control ($n = 34$)	0 (0 %)	18 (52.9 %)	16 (47.1 %)	0.26	0.74	<0.001	2.1859	1.4121–3.3838	<0.001	2.22	1.42–3.49
COPD ($n = 110$)	6 (5.5 %)	84 (76.4 %)	20 (18.2 %)	0.44	0.56						

Pearson's χ^2 test

n Number, *C* cytosine, *T* thymine, *CC* allele with cytosine–cytosine homozygote, *CT* allele with cytosine–thymine, heterozygote, *TT* allele with thymine–thymine homozygote, *OR* odds ratio, *CI* confidence interval

*P*1 value = 0.659 (normal versus COPD); *P*1 value < 0.001 (smoker control versus COPD); *P*2 value < 0.001 (normal versus smoker control)

Table 3 Association of COPD with polymorphisms of the TNF-α (−308) promoter

Groups	Genotypes			Allele frequency		*P* value	OR	95 % CI
	GG	GA	AA	G	A			
Normal ($n = 110$)	107 (97.3 %)	3 (2.7 %)	0 (0 %)	0.98	0.02	0.663	0.227	0.0516–0.9988
Control smoker ($n = 34$)	33 (97.9 %)	1 (3.1 %)	0 (0 %)	0.98	0.02	0.476	0.2079	0.0272–1.5882
COPD ($n = 110$)	99 (90 %)	11 (10 %)	0 (0 %)	0.95	0.05			

Pearson's χ^2 test

n Number, *G* guanine, *A* adenine, *GG* guanine–guanine, homozygote, *GA* guanine–adenine, heterozygote, *AA* adenine–adenine, homozygote, *OR* odds ratio, *CI* confidence interval

P value = 0.663 (control smoker versus normal); *P* value = 0.476 (smoker control versus COPD)

Table 4 Association of COPD with polymorphisms of the TNF-α (–238) promoter

Groups	Genotypes			Allele frequency		*P*1 value	OR1	95 % CI1	*P*2 value	OR2	95 % CI2
	GG	GA	AA	G	A						
Normal ($n = 110$)	83 (75.5 %)	13 (11.8 %)	14 (12.7 %)	0.81	0.19	0.002	4.352	2.1724–8.7186			
Smoker control ($n = 34$)	26 (76.5 %)	3 (8.8 %)	5 (14.7 %)	0.81	0.19	0.015	4.4909	1.9077–10.5718	0.865	1.0319	0.5159–2.0639
COPD ($n = 110$)	102 (92.7 %)	5 (4.5 %)	3 (2.7 %)	0.95	0.05						

Pearson's χ^2 test

n Number, *G* guanine, *A* adenine, *GG* guanine–guanine, homozygote, *GA* guanine–adenine, heterozygote, *AA* adenine–adenine, homozygote, *OR* odds ratio, *CI* confidence interval

*P*1 value = 0.002 (normal versus COPD); *P*1 value = 0.015 (smoker control versus COPD); *P*2 value = 0.865 (normal versus smoker control)

allele of COPD patients were higher than the control groups (Table 4).

Association Between Serum Levels of TNF-α and TGF-β1 with Different Severities of COPD

The serum TNF-α level in severe and very severe COPD patients was significantly higher compared with those with less severe disease (normal, mild, and moderate; Fig. 1a). Furthermore, the serum level of TNF-α of COPD with emphysema showed a trend to be higher than chronic bronchitis type (Fig. 1b). The significance of difference was as follows: (a) $P < 0.05$ compared with normal; (b) $P < 0.05$ compared with control smokers with control smokers; (c) $P < 0.05$ compared with mild COPD; (d) $P < 0.05$ compared with moderate COPD.

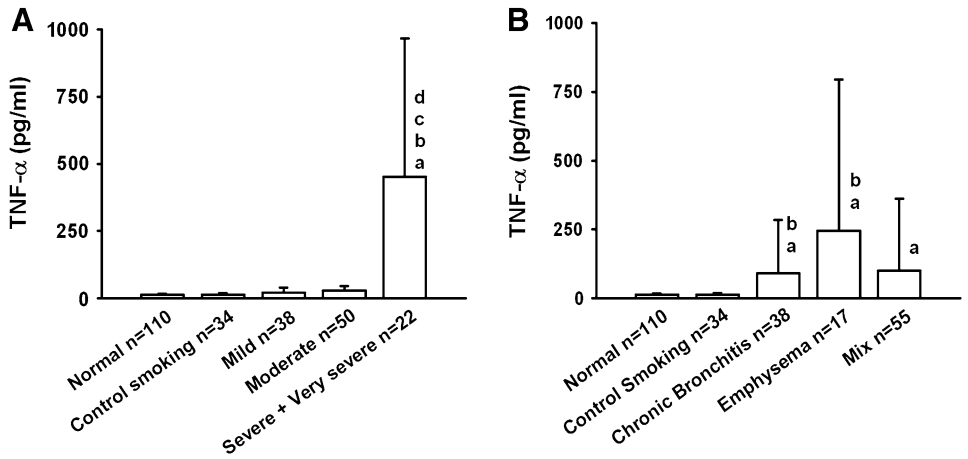

Fig. 1 The serum level of TNF-α of control and various severities of COPD groups

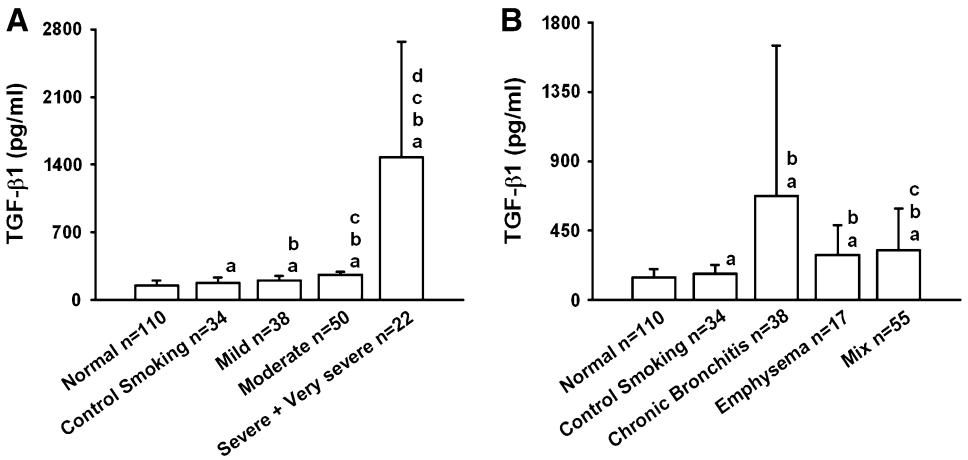

Fig. 2 The serum levels of TGF-β1 of control and various severities of COPD groups

The serum levels of TGF-β1 were significantly different among COPD patients with different disease severities (nonsmoker controls, control smokers, mild and moderate COPD; Fig. 2a). Serum level of TGF-β1 in mild COPD was higher than normal subjects. These results suggest TGF-β1 and TNF-α may modulate the severity of clinical phenotypes. Furthermore, TGF-β1 may be more sensitive than TNF-α for assessing the stages of COPD. In addition, the serum TGF-β1 level of COPD with chronic bronchitis had a trend higher than that in emphysema type (Fig. 2b). The significance of difference was as follows: (a) $P < 0.05$ compared with normal; (b) $P < 0.05$ compared with control smoker; (c) $P < 0.05$ compared with control smoker; (c) $P < 0.05$ compared with mild COPD (Fig. 2a) or chronic bronchitis (Fig. 2b); (d) $P < 0.05$ compared with moderate COPD respectively.

Interaction of Serum Levels of TNF-α and TGF-β1

All studied subjects were divided into four groups. We defined the high or low levels based on the higher serum value of TNF-α or TGF-β1 at the 95th percentile among controls with nonsmokers and smokers as cutoff value of TNF-α (17.56 ρg/ml) or TGF-β1 (224.35 ρg/ml). TNF-α or TGF-β1 more than cutoff value (17.56, 224.35 ρg/ml, respectively) was defined as "high." Comparison of these four groups has shown the FEV_1 of the group with high TGF-β1 + low TNF-α or low TGF-β1 + high TNF-α or high TNF-α + high TGF-β1 to be lower than the low TGF-β1 + low TNF-α group. However, the lowest FEV_1 was found in the group with high TNF-α + high TGF-β1 (Fig. 3).

Discussion

Our study found that polymorphisms of TNF-α has been associated with COPD. More severe clinical phenotype or airflow limitation in COPD patients was associated with higher serum levels of TGF-β1 and TNF-α. A high level of TGF-β1 existed in the mild stage of COPD. Combination of TGF-β1 and TNF-α may have additive effect on the severity of airflow limitation of COPD.

Previous studies of TGF-β1 and TNF-α polymorphisms have been conflicting with COPD. The association between

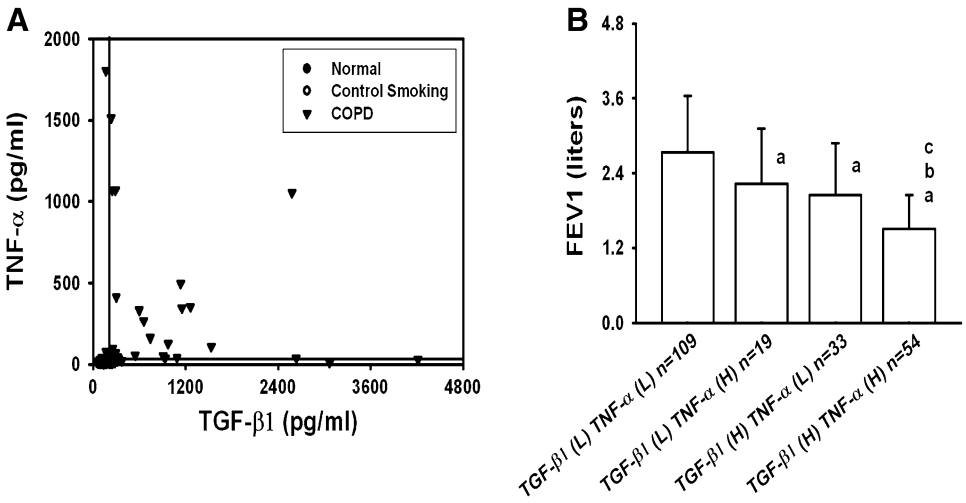

Fig. 3 All studied subjects (controls with smokers and nonsmokers and COPD) were divided into *four* groups and comparison of FEV_1 of various groups. We defined the *high or low* levels based on the *higher* value of TNF-α or TGF-β1 at the 95th controls as *cutoff* value of TNF-α (17.56 ρg/ml) or TGF-β1(224.35 ρg/ml). High value (H) was defined by *higher than cutoff* value and lower value (L) was defined by *lower than cutoff* value. **a** The four groups of studied subjects were divided into four groups by cutoff values of TNF-α and TGF-β1. **b** The value of FEV_1 in four groups: groups 1: TNF-α(L) + TGF-β1(L); group 2: TNF-α(H) + TGF-β1(L); group 3: TNF-α(L) + TGF-β1(H); and group 4: TNF-α(H) + TGF-β1(H) were compared. Group 1: TNF-α(L) + TGF-β1(L) contained 74 normal, 18 smoker control, and 17 COPD. Group 2: TNF-α(H) + TGF-β1(L) contained 5 normal control, 1 smoker control, and 13 COPD. Group 3: TNF-α(L) + TGF-β1(H) contained 3 normal, 4 control smoker, and 26 COPD. Group 4: TNF-α(H) + TGF-β1(H) contained 0 normal control, 0 smoker control, and 54 COPD. **b** The FEV_1 of groups 2 and 3 was lower than group 1 and the FEV_1 of group 4 was lower than those in groups 1, 2, and 3. *a–c* $P < 0.05$ compared with groups 1, 2, and 3, respectively

genetic polymorphism of the TNF-α −308 gene promoter with COPD remains controversial [14–16]. Our results suggest that TNF-α −308 gene promoter polymorphism were not associated with COPD risk, but we are the first to show genetic polymorphism TNF-α −238 G/A to be associated with COPD risk. This will need to be confirmed in further studies. The association between genetic polymorphism of TGF-β1 and COPD also is in contrast to the results of previous studies [17–19]. Our study showed that promoter gene C-509T TGF-β1 had no association with COPD. These conflicting results may have arisen from a range of factors, such as racial/ethnic differences, linkage or case-control association study, sample size, and differing diagnostic criteria of COPD. In our study, COPD patients were enrolled based on strict diagnostic criteria and were regularly followed in our outpatient clinic.

Our results are consistent with those of a previous study which found upregulation of TNF-α in severe COPD according to GOLD staging [23]. Furthermore, to demonstrate a correlation with phenotype, it can be defined by high-resolution chest CT, serum TNF-α level of chronic bronchitis, or emphysema patient who was higher than that in controls without COPD (either smoking or non-smoking). Moreover, TNF-α of emphysema type had a trend to be higher than in chronic bronchitis type and further study is required to investigate the different serum concentrations existing in various phenotypes and explore different endotypes based on different molecule pathways in various phenotype of COPD.

In a previous study, 63 patients with stable COPD (spirometric GOLD stages 2–4) and 17 controls were investigated and showed significantly elevated serum TGF-β1 levels in all COPD compared with controls, whereas the highest TGF-β1 serum level has been found only in spirometric GOLD stage 4 [24]. Our studies are in accordance with these findings; furthermore, we are the first to find serum TGF-β1 level of mild stable COPD to be higher than control smokers (non-COPD). These findings reflect a stage-dependent association with TGF-β1 in stable COPD. Different from TNF-α, TGF-β1 is upregulated in early-stage COPD, which reflects a more sensitive candidate as a serum biomarker to identify the severity of COPD. Moreover, we are the first to demonstrate the serum TGF-β1 level of chronic bronchitis type to trend higher compared with that of emphysema type. This issue is worth investigating what pathways play key roles in the development of emphysema and chronic bronchitis. TGF-β1 has a multitude of effects. In addition to possessing anti-inflammatory effect [25], TGF-β1 is a potent inductor of airway fibrosis and extracellular deposition of collagen [26]. Patients with COPD have increased expression of TGF-β1 in the airway epithelium, which has been associated with enhanced fibrotic airway remodeling [24, 27]. Other reports have postulated that increased TGF-β1 expression in COPD is predominantly

vessel-associated [28]. Our observation for the serum TGF-β1 levels supports the hypothesis that mediator plays an important role in airway remodeling of COPD.

Based on previous studies, both TGF-β1 and TNF-α appear to play key roles in the pathogenesis of COPD. However, no previous studies have investigated a relationship between these cytokines in clinical phenotypes and disease severities. Our study opens a new gateway in polymorphisms and severity association in COPD patients with following facts: 1) TGF-β1 is elevated in mild COPD than control smokers; 2) TGF-β1 are higher in chronic bronchitis than emphysema; and 3) combined higher level of both TNF-α and TGF-β1 has additive effects on airway obstruction as FEV_1.

The limitations of this study are the sample size of smoker (non-COPD) is relatively small and we did not explore the entire genetic polymorphisms of TNF-α and TGF-β1. Additional investigations are needed to explore the relevance of these genetic polymorphisms in COPD.

Conclusions

The genetic polymorphism of the TNF-α was associated with COPD. Both TGF-β1 and TNF-α serum levels were associated clinical severity and airflow limitation of COPD in an additive manner. Our results associating TGF-β1 and TNF-α with clinical severity of COPD suggest potential use of these parameters in the evaluation and management of COPD patients but this issue needs further investigation.

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Conflict of interest There is no conflict of interest for all authors.

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