Respiratory Medicine (2009) **103**, 1083–1089

available at www.sciencedirect.com

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journal homepage: www.elsevier.com/locate/rmed

Elevated plasma TGF-β_1 levels in patients with chronic obstructive pulmonary disease

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Received 2 December 2008; accepted 6 January 2009
Available online 31 January 2009

KEYWORDS
Chronic obstructive pulmonary disease;
Genetic polymorphism;
Plasma;
Transforming growth factor-β_1

Summary
Background: Transforming growth factor-β_1 (TGF-β_1), a multifunctional cytokine, has been implicated to be responsible for the increased deposition of extracellular matrix in the airways, and increased submucosal collagen expression in chronic obstructive pulmonary disease (COPD). We determined plasma TGF-β_1 levels in patients with COPD and explored its association with common functional polymorphisms of TGF-β_1 gene at C-509T and T869C in the development of COPD in a case–control study.
Methods: Stable COPD patients who were ever smokers, and age and pack-years smoked matched healthy controls ($n = 205$ in each group) were recruited for measurement of plasma TGF-β_1 levels using commercially available ELISA kit, and genotyped at C-509T and T869C functional polymorphisms of TGF-β_1 gene using polymerase chain reaction and restriction fragment length polymorphism (PCR–RFLP).
Results: COPD patients had significantly elevated plasma TGF-β_1 levels in comparison to healthy controls irrespective of the genotypes. Allele frequencies and genotype distributions at both polymorphic sites were not different among COPD patients or controls. TGF-β_1 levels were inversely correlated (Pearson's correlation analysis) with FEV_1 (% predicted) ($p < 0.001$) and FVC (% predicted) ($p < 0.001$).

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doi:10.1016/j.rmed.2009.01.005

Conclusion: The findings of elevated plasma TGF-β_1 levels in patients with COPD suggest that TGF-β_1 may play a role in COPD pathogenesis. The C-509T and T869C functional polymorphisms of TGF-β_1 gene do not represent a genetic predisposition to COPD susceptibility in Hong Kong Chinese patients.

Introduction

The prevalence of chronic obstructive pulmonary disease (COPD) is increasing all over the world, as is the mortality rate.[1] COPD is a slowly progressive and irreversible disorder characterized by the functional abnormality of airway obstruction due to chronic bronchitis, emphysema and/or small airways disease.[2] Cigarette smoking is the major risk factor, accounting for 80—90% of the COPD cases.[3] The risk of developing COPD among continuous smokers has been estimated to be 25%.[4]

Transforming growth factor-β_1 (TGF-β_1) is a multifunctional cytokine with many different effects on cell proliferation and differentiation and on inflammation[5] TGF-β_1 is implicated in several aspects of fibrosis, including the deposition of extracellular matrix proteins such as collagens and fibronectin.[6] Animal studies have shown that TGF-β_1 has an inhibitory effect on immunoglobulin synthesis by lymphocytes and can suppress airway hyperresponsiveness and inflammation.[7] Higher TGF-β_1 expression has been found in airway epithelium of smokers with COPD than in smokers without COPD, and in lung tissue from smokers with chronic bronchitis than from nonsmokers in some studies[8,9] but not in another study.[10] The data on the circulating TGF-β_1 between COPD patients and healthy controls have been inconsistent.[11,12] The production of TGF-β_1 has been reported to be under genetic control.[13,14] The TGF-β_1 gene is located on chromosome 19q13 and has been cloned and sequenced.[15] There are several registered polymorphisms within TGF-β_1 and its promoter that might be functional.[16] Two polymorphisms of the TGF-β_1 gene, C-509T and/or T869C, have been found to be associated with COPD in Caucasians and Chinese[17—19] but not in Korean[20] but correlation between plasma TGF-β_1 levels and the genotypes have not been carried out.

In the present study, we measured plasma TGF-β_1 levels in all patients with COPD and age-/pack years-matched healthy controls. We also genotyped and evaluated the association of TGF-β_1 gene at C-509T and T869C genetic variants regulating the expression of TGF-β_1 with the development of COPD in Hong Kong Chinese population.

Methods

Subjects

205 consecutive patients with COPD who were ex- or current smokers were recruited from outpatient respiratory clinics of 5 different hospitals across Hong Kong if they satisfied the clinical criteria of COPD according to the Global Strategy for Obstructive Lung Disease (GOLD) guidelines.[21] Exclusion criteria were history of asthma or other lung diseases. They all had postbronchodilator FEV_1 of $<$80% predicted, FEV_1/FVC of $<$70% and FEV_1 bronchodilator response of $<$12% and less than 200 ml with or without symptoms of cough, phlegm and shortness of breath on exertion. Each patient was matched for age ($\pm$5 years) and pack-years smoked ($\pm$5 years) with a healthy control without respiratory symptoms and with normal lung function ($FEV_1 \geq$ 80% predicted and FEV_1/FVC $\geq$ 70%) according to their smoking status. The predicted values were based on reference values obtained from the local population.[22] The healthy controls were recruited from the general population as described in a previous communication,[22] or from community centers and churches for the elderly in different parts of Hong Kong. In addition to lung function testing, these subjects were interviewed with a questionnaire for information on smoking habits, respiratory symptoms and other illnesses. Ever smokers included current smokers and ex-smokers who were defined as those who had not smoked within the last 12 months prior to the study.

Spirometry was carried out in all patients and controls using standardized methods according to the American Thoracic Society criteria.[23] In addition, a venous blood sample of 10 ml was taken from each participant. All patients and healthy controls were either born in Hong Kong or migrated from the Province of Guangdong, where Hong Kong is situated. The Ethics Committee of The University of Hong Kong has approved the study. Informed consent was obtained from each participant of the study.

Blood collection and storage

10 ml of whole blood in lithium heparin or in EDTA was taken from each subject and immediately centrifuged at 1600 $\times$ g for 10 min at 4 °C. Red blood cells, buffy coat and plasma were separated and stored at $-$70 °C until assayed.

Genotyping

DNA was extracted from buffy coats using commercially available DNA extraction kit (QIAGEN Inc.; Hilden, Germany). The genotypes of C-509T (rs1800469) and T869C (rs1982073) of TGF-β_1 gene were determined by polymerase chain reaction—restriction fragment length polymorphism (PCR—RFLP) as described previously.[24] The T869C variant was amplified using the GC-rich PCR system (Roche; Mannheim, Germany). The PCR products were digested with restriction enzyme DdeI for C-509T and BglI for T869C, respectively, and electrophoresed on 4% agarose gels. Samples were genotyped from the digested band sizes. The genotype of DNA samples was identified blindly and controls were prepared and set up in association with every single PCR operation as blank control (without DNA

template), positive control, and negative control. For any samples where amplification or digestion failed, analyses were repeated at least once.

TGF-β_1 measurement

Plasma was available for measurement of TGF-β_1 only in 174 patients and 183 healthy ever smokers. The biologically active TGF-β_1 concentration, after release from latent complexes by acidification, was determined using commercially available ELISA kit (BD OptEIA™ Set for human TGF-β_1; San Diego, CA, USA). Plasma samples were activated and diluted with assay buffer as manufacturer's instructions, bringing TGF-β_1 values of all the diluted samples within the linear range of the standard curve (62.5–4000 pg/ml). Final plasma TGF-β_1 concentrations were obtained by multiplication with the dilution factor. All samples from patients and controls were measured in adjacent wells to minimize assay variability. The reproducibility, calculated as the coefficient of variation (CV), was 7.5%.

Statistical analysis

Demographic data were compared for differences between groups using Student t test or χ^2 test whichever was appropriate. Data for plasma TGF-β_1 levels judged to be skewed by the Kolmogorov–Smirnov test were log-transformed and summarized using median (interquartile range). Differences in plasma levels of TGF-β_1 between patients and controls and between different genotypes were examined by Student t test.

Differences in genotype and allele frequencies between patients and controls were tested by using χ^2 analyses for a 2×2 table or by using the Fisher exact test. The odds ratios (OR) and 95% confidence intervals (CI) of COPD risk of individuals with various genetic polymorphisms were calculated using logistic regression analysis between COPD patients and healthy ever smokers. Pairwise linkage disequilibrium coefficients (D'), and Hardy–Weinberg equilibrium were estimated using SNPAnalyzer program (Istech Inc., Goyang-si, Korea; http://istech21.com/).

The significance of polymorphisms of various genotypes in relationship to the severity of COPD was also analyzed with patients categorized into severity groups by level of FEV_1 % predicted: $\geq$50%–79%, $\geq$30–49% and $<$30% according to the GOLD guidelines.

Pearson's correlation coefficients between plasma TGF-β_1 levels (log-transformed) and lung function were obtained. SPSS for Windows version 16.0 statistical package (SPSS, Chicago, IL) was used for statistical analyses. A p value of <0.05 was considered statistically significant.

Power calculation

The statistical power of the study sample sizes of cases and controls for detecting an association between the putative risk alleles of TGF-β_1 gene polymorphism and COPD was calculated with the Genetic Power calculator.[25] Given the frequencies observed in previous studies[17–20] for the putative risk alleles and assuming relative risks between 1.7 and 2.8, our case–control study has 80% power to detect significant differences at an α level of 5%.

Results

The characteristics of the study subjects are shown in Table 1. There were 205 pairs matched for age and packed years smoked. Among them, 19 COPD patients and 4 healthy controls were females. Compared with healthy ever smokers, COPD patients showed significantly lower FEV_1 (% predicted) and FVC (% predicted).

We found that plasma levels of TGF-β_1 were significantly elevated in COPD patients compared with healthy controls of the same genotypes (Table 2). However, we observed no significant differences in plasma levels of TGF-β_1 between different genotypes among healthy controls or COPD patients. There was a trend for plasma levels of TGF-β_1 to be higher among current smokers than ex-smokers in COPD patients or healthy controls (Fig. 1). Pearson's correlation analysis showed that plasma TGF-β_1 levels were inversely associated with FEV_1 (% predicted) (Fig. 2), and FVC (% predicted) ($p < 0.001$; Fig. 3) in our study population.

No differences were found in the distribution of genotypes and alleles of TGF-β_1 gene polymorphisms at C-509T and T869C in COPD patients and healthy controls (Table 3). The genotype distribution of both polymorphisms were consistent with the Hardy–Weinberg equilibrium and both polymorphisms at C-509T and T869C were in tight linkage disequilibrium ($D' = 0.9880$ and 0.9616 in COPD patients and healthy controls respectively). There were also no significant differences in the distribution of genotypes and alleles of TGF-β_1 gene polymorphisms at C-509T and T869C between healthy controls and COPD patients of varying severity (Table 4).

Discussion

Since only 25% of continuous smokers develop clinically significant airflow obstruction,[4] it has been suggested that individual susceptibility may be crucial in the pathogenesis of COPD. We demonstrated a negative correlation between circulating levels of TGF-β_1 and FEV_1 (% predicted), or FVC (% predicted) in our study population, supporting a possible role for TGF-β_1 in COPD pathogenesis. Several factors may contribute to TGF-β_1 overproduction in patients with COPD

Table 1 Characteristics of study subjects.

	Healthy controls	COPD patients
N	205	205
Gender (F/M)	4/201	19/186
Age (yrs)	66.9 ± 9.3	67.2 ± 8.5
Smoking status		
Current smokers	100	43
Ex-smoker	105	162
Pack-years smoked	36.7 ± 23.0	37.0 ± 22.9
FEV_1 % predicted[a]	101.3 ± 13.2	36.9 ± 15.4
FVC % predicted[a]	101.0 ± 12.1	69.4 ± 20.7

Means ± SD are shown.
[a] Differences between groups by t test.

Table 2 Plasma levels (pg/ml) of TGF-β_1 in age-/pack years-matched healthy ever smokers and patients with COPD according to genotypes.

	Healthy controls	COPD patients
C-509T		
Genotype		
CC	3064 (1952—4070) (36)	6125[a] (4317—8503) (33)
CT	3032 (1867—4254) (90)	7018[a] (4949—9557) (88)
TT	2362 (1761—3737) (68)	6482[a] (4750—8767) (65)
T869C		
Genotype		
TT	3340 (1971—4329) (39)	6128[a] (4327—8193) (34)
TC	2910 (1669—4222) (92)	7189[a] (4822—9583) (86)
CC	2377 (1830—3732) (63)	6482[a] (4923—8925) (65)

Medians (interquartile range) are shown.
[a] $p < 0.001$ for comparison between healthy controls and COPD patients among ever smokers.

including genetics. However, we found no significant differences in the distribution of genotypes and alleles of TGF-β_1 gene polymorphisms at C-509T and T869C between COPD patients and controls matched for age and pack years smoked. The lack of association of C-509T and/or T869C polymorphisms of TGF-β_1 gene in COPD patients in our population was contrary to that reported among Caucasians in the United States, New Zealand and in other Chinese populations,[17—19] but in agreement with that reported in a Korean population.[20] We also found no association between TGF-β_1 gene polymorphisms at C-509T and T869C, and severity of airflow obstruction in our COPD patients as reported in a recent study.[24]

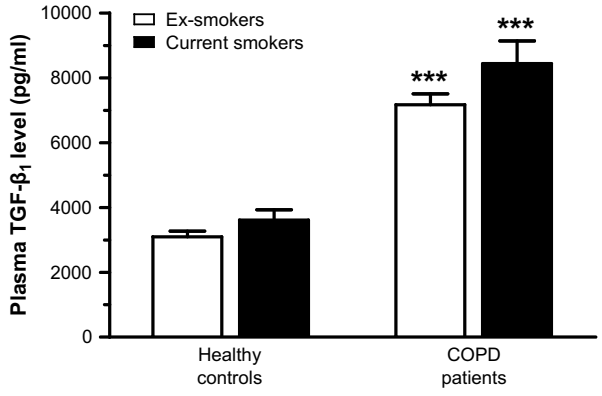

Figure 1 Plasma TGF-β_1 level in all age-/pack years-matched healthy controls according to smoking status ($n = 105$ ex-smokers and 99 current smokers respectively) and COPD patients ($n = 149$ ex-smokers and 40 current smokers respectively). Means ± SEM are shown. ***$p < 0.001$ for comparison of ex-smokers or current smokers between COPD patients and healthy controls respectively.

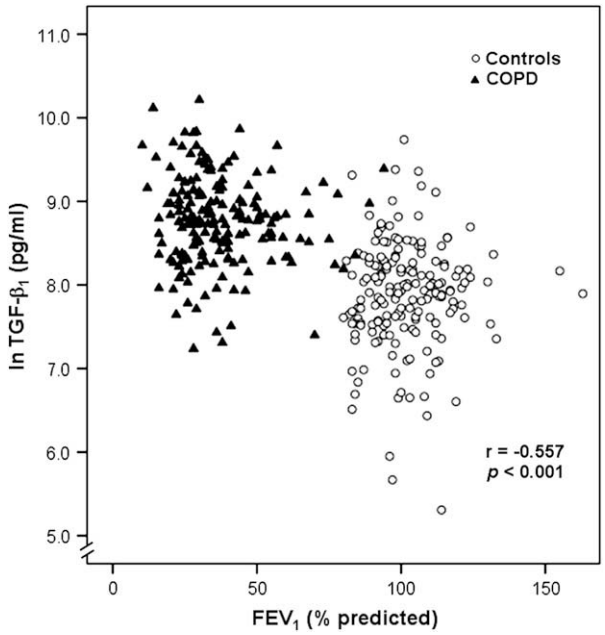

Figure 2 Correlation between FEV_1 (% predicted) and plasma TGF-β_1 concentration. Pearson's correlation coefficient (r) is given.

Both C-509T and T869C polymorphisms investigated in this study have been reported to be associated with the transcriptional activity of the gene or the serum level of the gene product. The CC genotype of C-509T polymorphism was associated with significantly lower circulating level of TGF-β_1 compared with other genotypes in Caucasian women and Indian populations[15,26] while the CC genotype of the T869C polymorphism was associated with higher

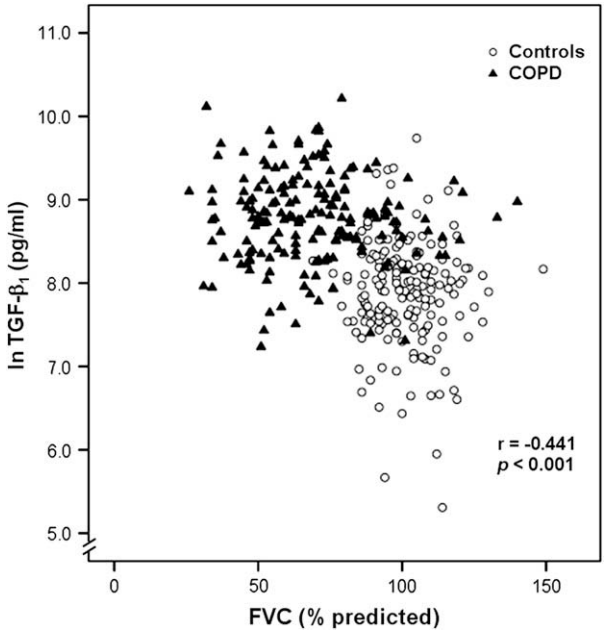

Figure 3 Correlation between FVC (% predicted) and plasma TGF-β_1 concentration. Pearson's correlation coefficient (r) is given.

Table 3 Genotype distributions and allele frequencies of C-509T and T869C polymorphisms of TGF-β_1 gene in healthy controls and COPD patients.

	Healthy controls, *N* (%)	COPD patients, *N* (%)	*p* value
C-509T			
Genotype			
CC	36 (18.5)	35 (17.3)	0.923
CT	90 (46.1)	97 (48.0)	
TT	69 (35.4)	70 (34.7)	
Allele			
C	162 (41.5)	167 (41.3)	1.000
T	228 (58.5)	237 (58.7)	
T869C			
Genotype			
TT	39 (20.0)	38 (18.9)	0.817
TC	93 (47.7)	92 (45.8)	
CC	63 (32.3)	71 (35.3)	
Allele			
T	171 (43.8)	168 (41.8)	0.566
C	219 (56.2)	234 (58.2)	

TGF-β_1 concentration than other genotypes in a Japanese study.[27] We were unable to find such associations, similar to one previous report in Caucasian.[28]

TGF-β_1 induces a wide diversity of biological processes that regulate growth and differentiation of cells, tissue repair, and extracellular matrix component production. Using an animal model, one study reported abnormalities in the activation and signaling of TGF-β_1 to be important in the pathogenesis of emphysema.[29] Our data do not permit us to determine whether TGF-β_1 overproduction is the cause or consequence of COPD. In this study, COPD patients had higher plasma levels of TGF-β_1 compared with healthy controls irrespective of whether they were current smokers or ex-smokers, suggesting that this could be the result of inflammatory process associated with COPD and TGF-β_1 might be released by the inflammatory cells. As the disease progresses and airflow obstruction increases, TGF-β_1 is being produced in larger amounts in activated macrophages in response to tissue injury and accumulates in the circulation. The findings of higher TGF-β_1 expression in airway epithelium of smokers with COPD than in smokers without COPD, and in lung tissue from smokers with chronic bronchitis than from nonsmokers[8,9] are in support of such a hypothesis although, Kokturk et al.[10] were not able to confirm the increased expression of TGF-β_1 in biopsy of the airway in patients with COPD. On the other hand, elevated TGF-β_1 level from cigarette smoking may stimulate tissue remodeling responses and may lead to emphysema and/or fibrosis in the injured lung. Rahman and co-workers[30] have found correlation between increased TGF-β_1 expression and immunoreactivity for 4-hydroxy-2-nonenal, a marker of oxidative stress in peripheral lung tissue of COPD patients. TGF-β_1 is secreted in a latent form that is inactive but is potently activated by MMP-9.[31] MMP-9$^{-/-}$ mice are not protected against emphysema induced by cigarette smoke, but they are protected from small airway fibrosis.[32] Furthermore, studies on the differential expression of genes between normal smokers and COPD patients at GOLD stage 2 revealed marked elevation of TGF-β_1.[33]

For the majority of multifactorial diseases, such as COPD, it is unlikely that a single polymorphism in a single gene could alter expression or function of specific protein to produce pathologic phenotypes but rather the result of combined effects of different single nucleotide polymorphisms (SNPs) in a gene leading to changes in expression/function. The G915C polymorphism located within exon 1 at codon 25 has been found to be associated with

Table 4 Genotype frequencies of C-509T and T869C polymorphisms of TGF-β_1 gene in healthy controls and COPD patients classified according to severity of airflow obstruction.

Polymorphic sites	Healthy controls, *N* (%)	COPD patients, *N* (%)			*p* value
	FEV_1	FEV_1	FEV_1	FEV_1	
	$\geq$80%	$\geq$50—79%	$\geq$30—49%	<30%	
C-509T					
Genotype					
CC	36 (18.5)	7 (18.9)	14 (16.5)	13 (17.6)	0.851
CT	90 (46.1)	20 (54.1)	43 (50.6)	31 (41.9)	
TT	69 (35.4)	10 (27.0)	28 (32.9)	30 (40.5)	
Allele					
C	162 (41.5)	34 (45.9)	71 (41.8)	57 (38.5)	0.765
T	228 (58.5)	40 (54.1)	99 (58.2)	91 (61.5)	
T869C					
Genotype					
TT	39 (20.0)	8 (19.3)	14 (21.1)	15 (20.5)	0.867
TC	93 (47.7)	19 (40.9)	40 (45.3)	30 (41.1)	
CC	63 (32.3)	10 (39.8)	31 (33.6)	28 (38.4)	
Allele					
T	171 (43.8)	35 (47.3)	68 (40.0)	60 (41.1)	0.682
C	219 (56.2)	39 (52.7)	102 (60.0)	86 (58.9)	

Table 5 Power calculations for a sample size of $n = 205$ ever smoking patients with COPD and 205 healthy ever smokers with $\alpha = 0.05$ and $\beta = 0.80$ for a two-sided test.

Prevalence of polymorphism[a]	Minimum detectable relative risk
5%	2.8
10%	2.2
20%	1.9
30%	1.8
40%	1.7
50%	1.7

[a] Individuals who have either one or two copies of the putative risk allele.

decreased production of TGF-β_1 *in vitro* (C allele), increased frequency of lung fibrosis and a more rapid decline of lung function in patients with cystic fibrosis.[34] We have not yet studied this polymorphism because the variability at this site has not been reported in populations from Korea, Japan and China.[35–37]

In this study, we performed not only plasma TGF-β_1 measurement but also genotype analysis to determine whether TGF-β_1 gene polymorphisms at C-509T and T869C were associated with functional activity such as regulation of its expression. Our cases and controls are well matched for age and pack-years smoked to avoid the confounding effect since serum levels of TGF-β_1 was found to be inversely correlated with age and positively correlated with smoking in healthy Japanese individuals.[38] We included subjects of Chinese ethnic origin from Hong Kong or from the Province of Guangdong where Hong Kong is situated, to avoid ethnic differences. The sample size was moderate, greater than three previous studies,[18–20] but less than the others.[17,24] Power calculation showed that our sample size of 205 in each group should be adequate to detect relative risks in the range of 1.7–2.8 (Table 5). There are limitations to our study. The measurement of TGF-β_1 levels had been carried out only in plasma, which might not reflect the local levels in the lungs. Other biological samples such as BAL, induced sputum and exhaled breath condensate, might be more appropriate. Additionally we measured TGF-β_1 level at a single time point, which ignores any variability in this parameter over time.

In conclusion, we found that polymorphisms of TGF-β_1 gene at C-509T and T869C are not related to plasma TGF-β_1 levels and are not associated with increased susceptibility to development of COPD from cigarette smoking in Hong Kong Chinese population. However, we found elevation of plasma TGF-β_1 levels in patients with COPD which were negatively correlated with lung function parameters (FEV_1 or FVC % predicted), suggesting that TGF-β_1 may be involved in the pathogenesis. Further studies are required to confirm this finding and to determine its role in the disease process.

Conflict of interest

None of the authors have a conflict of interest to declare in relation to the contents of this paper.

Acknowledgements

This work was supported in part by a grant from CRCG Seed Funding for Basic Research of the University of Hong Kong and partly by the Hong Kong Lung Foundation. The authors wish to thank all nurses and laboratory staffs who took part in this study; all of the subjects for their participation; Anne DyBuncio, Occupational and Environmental Lung Diseases Unit, Department of Medicine, The University of British Columbia, who conducted statistical analysis of the data.

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