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The transforming growth factor-β1 (TGFB1) gene is associated with chronic obstructive pulmonary disease (COPD)

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Although cigarette smoking is the primary environmental risk factor, genetic risk factors likely influence the development of chronic obstructive pulmonary disease (COPD). Linkage analysis between short-tandem repeat markers on chromosome 19 and COPD phenotypes was followed by association analysis of single nucleotide polymorphisms in a gene on chromosome 19q [transforming growth factor-β1 (TGFB1)] and COPD phenotypes in a family-based sample and a case–control study (cases with severe COPD and control subjects with significant history of smoking but no COPD). Stratification by smoking status substantially improved the evidence of linkage to chromosome 19q for COPD phenotypes. Among former and current smokers in the Boston Early-Onset COPD Study, there was significant evidence of linkage between chromosome 19q and pre-bronchodilator (pre-BD) FEV_1 (LOD = 3.30) and suggestive evidence of linkage between chromosome 19q and other COPD phenotypes. In these families, a SNP in the promoter region of TGFB1 (rs2241712) and two SNPs in the 3′ genomic region of TGFB1 (rs2241718 and rs6957) were significantly associated with pre- and post-BD FEV_1 ($P < 0.05$). Among smokers in the COPD cases and control subjects, two SNPs in the promoter region of TGFB1 (rs2241712 and rs1800469) and one SNP in exon 1 of TGFB1 (rs1982073) were significantly associated with COPD ($P \leq 0.02$ in all cases). Chromosome 19q likely contains a genetic locus (or loci) that influences COPD through an interaction with cigarette smoking. We hypothesize that genetic variants in or near the TGFB1 gene influence the pathogenesis of COPD among cigarette smokers.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD), the fourth leading cause of death in the USA (1), is characterized by airflow obstruction due to chronic bronchitis, emphysema and/or small airways disease (2).

To study genetic determinants of COPD unrelated to severe alpha 1-antitrypsin (AAT) deficiency (3), we previously performed genome-wide linkage analysis for COPD phenotypes in extended pedigrees in the Boston Early-Onset COPD Study (4–6). To follow up on these results, we genotyped additional short tandem repeat (STR) markers on chromosome

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19 and performed linkage analysis for COPD phenotypes. On the basis of the significant linkage results, we performed analyses of association between single nucleotide polymorphisms (SNPs) in a candidate gene on chromosome 19 [transforming growth factor-β1 (TGFB1)] and COPD phenotypes in two independent populations, the first comprising members of families in the Boston Early-Onset COPD Study and the second comprising a group of individuals with severe COPD enrolled in the National Emphysema Treatment Trial (cases) (7) and a group of smokers without COPD enrolled in the Normative Aging Study (control subjects) (8).

RESULTS

The results of the linkage analysis between chromosome 19 and COPD phenotypes are shown in Table 1 and Figure 1. Among all subjects, the addition of STR markers in chromosome 19 modestly improved the evidence of linkage for all but one COPD phenotype. In this analysis, there was suggestive evidence of linkage (9) between chromosome 19 and three COPD phenotypes. After excluding the phenotypic data of non-smokers from the original genome-wide analysis, there was markedly increased evidence of linkage between chromosome 19 and pre-BD FEV_1. Among smokers, the addition of STR markers resulted in increased evidence of linkage between chromosome 19 and all but one COPD phenotype. In this smokers-only analysis, there was significant evidence of linkage between chromosome 19 and pre-BD FEV_1 (LOD = 3.30) and suggestive evidence of linkage between chromosome 19 and post-BD FEV_1 (LOD = 2.75). There was also suggestive evidence of linkage between chromosome 19 and three COPD phenotypes. Since most subjects with airflow obstruction were smokers, the results of the linkage analysis of qualitative COPD phenotypes among smokers were only minimally different from those among all subjects.

The TGFB1 gene is located on chromosome 19q13.2, between the genotyped STR markers D19S422 (at 63.1 cM) and D19S420 (at 66.3 cM), near our linkage peak for FEV_1 (Fig. 1). For the analysis of association between TGFB1 and COPD phenotypes, we selected two SNPs previously associated with obstructive airway diseases (10,11) and three additional SNPs that had primers available as TaqMan Assays-on-Demand from ABI. Among all participants in the Boston Early-Onset COPD Study, there was a significant association between three SNPs in or near TGFB1 [one in the promoter region (NCBI code: rs2241712) and two in the 3′ genomic region (NCBI codes: rs2241718 and rs6957)] and both pre-BD FEV_1 (Table 2) and post-BD FEV_1 ($P < 0.05$ in all cases). Two of these three SNPs (rs2241718 and rs6957) were also associated with pre-BD qualitative COPD phenotypes (Table 2) and with post-BD moderate to severe airflow obstruction ($P < 0.05$ in all cases). In addition, SNP rs6957 was associated with post-BD FEV_1/FVC ($P = 0.04$). These results were not appreciably changed after restricting the analysis to smokers only (Table 2). Two SNPs in TGFB1 (rs1982073 and rs1800469) were not significantly associated with any COPD phenotype.

Among the NAS and NETT subjects, only three of the 44 SNPs in the stratification panel were found to be differentially distributed at $P < 0.05$. Overall, there was no significant evidence of population stratification between NETT and NAS subjects ($\chi^2_{44} = 58.5$, $P > 0.05$).

Table 3 shows the results of the case–control analysis of the association between SNPs in TGFB1 and COPD among participants in the NAS and the NETT. Among the NAS (control) subjects, the five SNPs tested were in Hardy–Weinberg equilibrium ($P > 0.10$ in all instances). Under the assumption of an additive model of inheritance, there was a significant association between three SNPs in TGFB1 [two in the promoter region (rs2241712 and rs1800469) and one in exon 1 (rs1982073)] and COPD. These results were not appreciably changed after excluding the 110 women participating in NETT from the analysis (Table 3).

There was tight LD between the two SNPs in the promoter region of TGFB1 among NAS ($r^2 = 0.94$) and NETT subjects ($r^2 = 0.97$). There was also strong LD between the two SNPs in the 3′ genomic region of TGFB1 in NAS ($r^2 = 0.98$) and NETT ($r^2 = 0.99$) subjects. There was no LD between the SNPs in the 3′ genomic region and the other SNPs in TGFB1 in NAS or NETT subjects ($r^2 < 0.02$ in all cases). There was at least moderate LD between the SNP in exon 1 and the two SNPs in the promoter region of TGFB1 in NAS and NETT subjects ($r^2 > 0.60$ in all cases). The global test of association between the five haplotypes in TGFB1 with frequency >2% and COPD was significant ($\chi^2_5 = 11.5$, $P = 0.04$). Among all subjects, the most common haplotypes in TGFB1 were: (a) rs2241712 = A, rs1800469 = C, rs6957 = A, rs1982073 = T, rs2241718 = C (frequency = 0.48); and (b) rs2241712 = G, rs1800469 = T, rs1982073 = C, rs6957 = A and rs2241718 = C (frequency = 0.25). These two individual haplotypes were associated with COPD [$P = 0.007$ for haplotype (a) and $P = 0.04$ for haplotype (b)].

DISCUSSION

Because the major environmental risk factor for COPD (cigarette smoking) is known and readily quantifiable, we were able to assess a potential interaction between genes located in a genomic region linked to COPD phenotypes (chromosome 19) and cigarette smoking. Among former and current smokers in the Boston Early-Onset COPD Study, we found significant evidence of linkage between chromosome 19 and a critical COPD phenotype (FEV_1) and suggestive evidence of linkage between chromosome 19 and other COPD phenotypes. Our findings strongly suggest that there is a genetic locus (or loci) on chromosome 19 that interacts with cigarette smoking in the pathogenesis of COPD.

TGFB1, a gene located near the maximum observed LOD score for FEV_1, is an excellent candidate gene for COPD. Among participants in the Boston Early-Onset COPD Study, we found an association between three SNPs in or near TGFB1 (one in the promoter and two in the 3′ genomic region) and COPD phenotypes. In an independent comparison of white smokers with COPD and control subjects, we found a significant association between three SNPs in TGFB1 (one in exon 1 and two in the promoter region) and COPD. The 30 kb region surrounding TGFB1 includes two genes of unknown function (interim HUGO names: MGC2055 and MGC4093)

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Table 1. Multipoint analysis of linkage between short tandem repeat markers on chromosome 19 and COPD phenotypes in the Boston Early-Onset COPD Study

Phenotype	Maximum multipoint LOD score (location in cM)			
	All subjects[a]		Former and current smokers only[b]	
	Original scan	Additional markers included	Original scan	Additional markers included
Before administration of bronchodilator				
FEV_1	1.40 (59)	1.73 (62)	2.37 (71)	3.30 (63)
FEV_1/FVC	1.47 (61)	1.70 (62)	1.84 (64)	1.96 (62)
Mild to severe airflow obstruction[c]	1.38 (42)	0.75 (42)	1.45 (42)	1.02 (42)
Moderate to severe airflow obstruction[d]	2.12 (42)	2.27 (36)	1.84 (42)	1.87 (36)
After administration of bronchodilator				
FEV_1	1.91 (78)	2.14 (77)	1.44 (66)	2.75 (63)
FEV_1/FVC	1.47 (75)	1.87 (62)	1.83 (63)	2.15 (64)
Mild to severe airflow obstruction[c]	2.05 (42)	1.49 (42)	2.16 (42)	1.39 (42)
Moderate to severe airflow obstruction[d]	1.83 (42)	2.41 (43)	1.78 (42)	2.22 (36)

[a]Phenotypic data for 582 and 561 individuals were included in the linkage analysis of COPD phenotypes measured before and after administration of bronchodilator, respectively.
[b]Phenotypic data for 407 and 391 individuals were included in the linkage analysis of COPD phenotypes measured before and after administration of bronchodilator, respectively.
[c]$FEV_1 < 80\%$ of predicted and $FEV_1/FVC < 90\%$ of predicted.
[d]$FEV_1 < 60\%$ of predicted and $FEV_1/FVC < 90\%$ of predicted.
All of the analyses adjusted for sex, age, height and pack-years of cigarette smoking.

(12); a search of the UniGene database (http://www.ncbi.nlm.nih.gov/UniGene) revealed that transcripts corresponding to both of these genes have been identified in various cDNA libraries of lung tissue. Because two of the SNPs associated with COPD phenotypes in the Boston Early-Onset COPD Study (rs6957 and rs2241718) are located 3′ genomic to TGFB1 (within the 3′ UTR region of MGC20255), we cannot make firm conclusions regarding which gene(s) in this genomic region is a susceptibility gene for COPD. However, data from the International HapMap consortium suggest that the genomic region including TGFB1, MGC20255, and MGC4093 is organized in two large haplotype blocks in Caucasians (HapMap public release number 7) (13). The first block spans ∼26 kb and includes all of MGC4093 and the promoter and exon 1 of TGFB1; one of the SNPs examined in our study (rs1800469) was used to characterize the block structure in the HapMap project. The second block spans ∼105 kb and includes the two terminal exons of TGFB1, all of MGC20255, and two additional genes [heterogeneous nuclear ribonucleoprotein U-like 1 (HNRPUL1) and AXL receptor tyrosine kinase (AXL)]. Although we did not genotype any of the SNPs that the HapMap project used to characterize this second block, it is likely that SNPs rs2241718 and rs6957 are part of this block. Thus, we have evidence that SNPs in both haplotype blocks in the genomic region including TGFB1, MGC20255 and MGC4093 are associated with COPD phenotypes. Because only TGFB1 spans both of these haplotype blocks, TGFB1 is the most likely candidate to be a susceptibility gene for COPD.

In vitro, TGF-β1 regulates immune responses, growth and differentiation of cells, tissue repair, and extracellular matrix production (14,15). Studies of animal models strongly suggest that abnormalities in the activation and signaling of TGF-β1 are important in the pathogenesis of emphysema (16,17). Mice lacking integrin-mediated activation of latent TGF-β (Itgb6-null) develop pulmonary emphysema through alterations of a macrophage metalloelastase (MMP12) (16). In that experimental model, the effects of Itgb6 deletion were overcome by transgenic expression of versions of the β6 integrin subunit that support TGF-β or by simultaneous transgenic expression of active TGF-β1, suggesting that a functional alteration in TGF-β1 activation results in an increased risk of emphysema. Mice homozygous for a disrupted allele of an isoform of the latent TGF-β binding protein (LTBP4) develop reduced deposition of TGF-β in the extracellular space and severe pulmonary emphysema (17). In humans, the T allele of a SNP in the promoter region of the TGFB1 gene (-509 C to T, rs1800469) has been associated with phenotypes related to asthma (an obstructive airway disease) in some studies (11) but not others (18). This allele alters a Ying Yang 1 (YY1) transcription-factor consensus-binding site, enhances YY1 binding and promoter function, and is associated with higher circulating concentrations of TGF-β1 (11,19). We found an inverse association between the T allele of SNP rs1800469 and COPD in our population-based case–control study. The C allele of a SNP in exon 1 of TGFB1 (29 T to C) results in an amino acid substitution at codon 10 (Leu → Pro) and increased production of TGF-β1 (20). More recently, Wu *et al.* (10) showed an inverse association between the C allele of this SNP (rs1982073) and COPD among smokers, a finding that was replicated in our case–control study but not in our family-based study.

The main strengths of our study include focusing on families of probands with severe, early-onset COPD that may be enriched for genetic determinants of COPD, selection of a candidate gene on the basis of both positional and functional information, careful definition of COPD phenotypes in the Boston Early-Onset COPD Study and of severe emphysema in the NETT, demonstration of lack of significant population stratification among cases and control subjects, and

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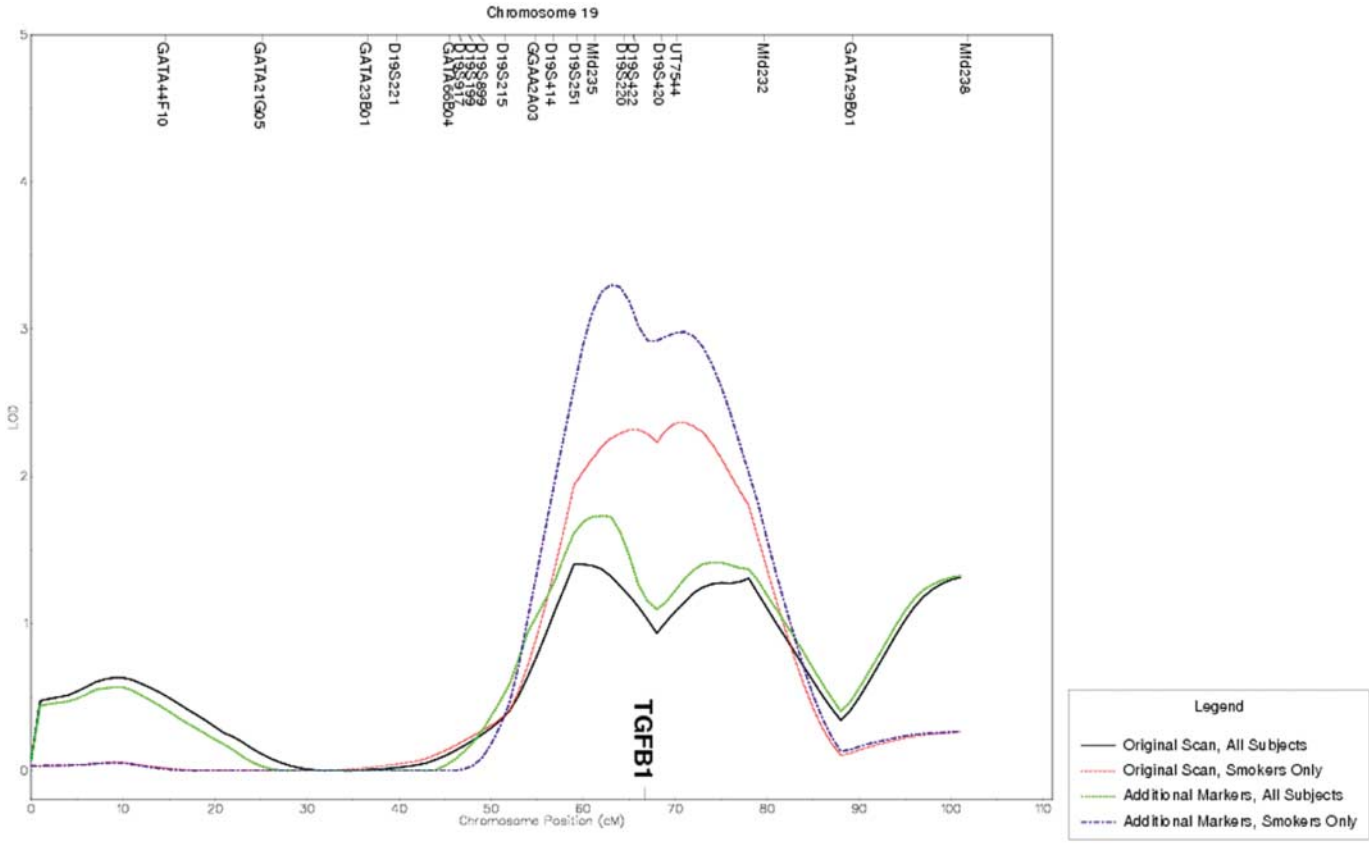

Figure 1. Results of the analysis of linkage between short-tandem repeat (STR) markers on chromosome 19 and pre-bronchodilator FEV_1 for the original genome scan and after inclusion of additional STR markers. The analysis was initially conducted among all subjects and then repeated among former and current smokers. The inclusion of additional markers increased the evidence of linkage. Stratification by smoking status also increased the evidence for linkage, with a maximum multipoint LOD of 3.30 including flanking STR markers. The TGF-β1 gene (TGFB1) is located near the linkage peak, between markers DS420 and DS422.

demonstration of global haplotypic association between TGFB1 and COPD. The main limitations of this study are the inclusion of a limited number of SNPs in TGFB1, lack of functional information on some of the SNPs included in the study and the absence of genotypic information on other genes in the candidate genomic region on chromosome 19. Although variance component linkage analysis results can be influenced by non-normality of the phenotype distributions, none of our phenotypes demonstrated kurtosis >2 (a threshold above which variance components linkage results can be biased) (21). Furthermore, we have previously shown no appreciable difference in our variance component linkage analysis results in the Boston Early-Onset Study using empirical *P*-values (6). Because we decided *a priori* that we would attempt to replicate the results of our analysis of association between variants in TGFB1 and COPD phenotypes in the Boston Early-Onset COPD Study in an independent population, we did not adjust for multiple comparisons in our association analysis.

In conclusion, chromosome 19q likely contains a genetic locus (or loci) that influences COPD through an interaction with cigarette smoking. We found an association between variants in a gene on chromosome 19q (TGFB1) and COPD phenotypes among smokers in two independent populations. We hypothesize that abnormal function of the TGFB1 gene is implicated in the pathogenesis of COPD among smokers. Further work will be required to determine whether functional variants in or near the TGFB1 gene can be identified.

MATERIALS AND METHODS

Study populations

Three populations were examined in this study: a group of families in the Boston Early-Onset COPD Study, a group of individuals without evidence of airflow obstruction enrolled in the Normative Aging Study (NAS), and a group of individuals with severe COPD enrolled in the National Emphysema Treatment Trial (NETT). Respective institutional review boards for human studies approved the study protocols and informed consent was obtained from all of the subjects enrolled in the three studies.

The recruitment, assessment and characteristics of the members of the families enrolled in the Boston Early-Onset COPD Study have been reported (22). In brief, ascertainment criteria for probands with severe, early-onset COPD included an FEV_1 equal to or lower than 40% of predicted, age lower than 53 years and absence of severe AAT deficiency. All available first-degree relatives, older second-degree relatives and 49 additional relatives of the ascertained COPD probands

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Table 2. Family-based analysis of association between single nucleotide polymorphisms (SNPs) in the TGFB1 gene and COPD phenotypes among individuals in the Boston Early-Onset COPD Study

Phenotype	*P*-values for FBAT statistic for TGFB1 SNPs[a]					
	rs2241718		rs2241712		rs6957	
	All	Smokers only	All	Smokers only	All	Smokers only
Before administration of bronchodilator						
FEV_1	0.03[d]	0.03[d]	0.03[d]	NS	0.03[d]	0.02[d]
FEV_1/FVC	NS	NS	NS	NS	NS	NS
Mild to severe airflow obstruction[b]	0.04[d]	0.04[d]	0.05[d]	NS	0.04[d]	0.04[d]
Moderate to severe airflow obstruction[c]	0.04[d]	0.04[d]	NS	NS	0.04[d]	0.04[d]

[a]Phenotypic data for 582 individuals were included in the association analysis of COPD phenotypes in all subjects. Phenotypic data for 407 individuals were included in the association analysis of COPD phenotypes in former and current smokers.
[b]$FEV_1 < 80\%$ of predicted and $FEV_1/FVC < 90\%$ of predicted.
[c]$FEV_1 < 60\%$ of predicted and $FEV_1/FVC < 90\%$ of predicted.
[d]Dominant model; all models adjusted for age, sex, height and pack-years of cigarette smoking.
Smokers only = former and current smokers; FBAT = family-based association test; NS = $P > 0.05$.

were invited to participate. The original genome-wide linkage analysis of COPD phenotypes included 585 individuals (582 with pre-bronchodilator spirometry) in 72 pedigrees with mean size of 8.1 individuals (range 2–18) (4,5). The mean (±SD) age of the 72 probands was 47.7 ± 5.3 years. Of the 585 individuals included in the original genome-wide linkage analysis of COPD phenotypes, 561 subjects had information on post-bronchodilator (post-BD) measures and were thus included in the genome-wide linkage analysis of post-BD spirometric measures.

Participants in the Boston Early-Onset COPD Study were predominantly white, but there were 10 African-American subjects. Each study participant completed a modified version of the 1978 ATS-DLD questionnaire (23) and performed spirometry as previously described (22). Spirometry was repeated approximately 15 min after albuterol administration. Although absolute-volume spirometric measurements were the primary quantitative COPD-related phenotypes for the analysis, spirometric values were also expressed as percentage of predicted values by use of equations formulated for white (24–26) and African-American (27) participants.

The National Emphysema Treatment Trial (NETT) is a randomized, multicenter clinical trial designed to compare lung volume reduction surgery and conventional medical therapy for severe emphysema. All of the subjects participating in NETT were selected on the basis of FEV_1 values below 45% predicted, evidence of hyperinflation in pulmonary function testing and bilateral emphysema on high resolution chest CT scan (7). This study included 304 white individuals participating in the ancillary genetics study of NETT to serve as COPD cases for the case–control analysis. These 304 subjects had a mean age (±SD) of 67.3 (±6.0) years and were all former or current smokers [mean (±SD) pack-years of cigarette smoking = 67.4 (±31.6)]; 194 (63.8%) of these individuals were men.

The Normative Aging Study (NAS) is a longitudinal study of aging conducted by the Veterans Administration (8). The study cohort was screened to exclude chronic health conditions and consisted of 2280 community-dwelling men from the Greater Boston area who were 21–80 years of age at the time of entry into the study between 1961 and 1969. Beginning in 1984, subjects have been studied with a questionnaire including questions on respiratory symptoms and smoking habits and pulmonary function testing. From the NAS subjects with information on respiratory health and pulmonary function testing, we selected 441 white men without evidence of airflow obstruction [$FEV_1 > 80\%$ of predicted (28) and $FEV_1/FVC > 90\%$ of predicted (20)] at their most recent visit and a history of ≥10 pack-years of cigarette smoking [mean (±SD) = 38.5 (±26.5) pack-years] to serve as control subjects for the case–control analysis. The mean age (±SD) of these individuals was 67.5 (±8.5) years.

Genotyping

Following the previously reported genome-wide linkage analysis in the Boston Early-Onset COPD Study (4–6), we genotyped an additional 10 STR markers on chromosome 19 in the 72 pedigrees included in the genome scan. For these additional markers, map locations were determined on the basis of the Marshfield map (http://research.marshfieldclinic.org/genetics/Map_Markers/maps), with confirmation from the UCSC Human Genome Project Working Draft (http://genome.ucsc.edu). Fluorescent-labeled and unlabeled primers were obtained from Research Genetics and Applied Biosystems (ABI, Foster City, CA). PCR was performed with Taq Gold Polymerase (ABI) in MJ Research PCR machines. Product sizes were assessed on an ABI 3100 machine. GENESCAN and GENOTYPER version 3.7 software were used to assist with genotype determination. Linkage analysis was performed with 10 markers (GATA44F10, GATA21G05, GATA23B01, GATA66B04, GGAA2A03, Mfd235, UT7544, Mfd232, GATA29B01 and Mfd238) from the original genome scan and 10 additional STR markers located at 33–68 cM on chromosome 19 (D19S199, D19S215, D19S220, D19S221, D19S251, D19S414, D19S420, D19S422, D19S899, D19S917). The genotype completion rates for both the markers in the original genome scan and the additional markers was >97%. The PEDCHECK program was used to test for Mendelian inconsistencies at individual markers in the 585 genotyped individuals (29).

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Table 3. Analysis of association between single nucleotide polymorphisms (SNPs) in the TGFB1 gene and severe COPD among participants in the National Emphysema Treatment Trial (NETT) and the Normative Aging Study (NAS)

SNPs	Locus	Allelic frequencies									
		NAS subjects (controls)				NETT subjects (cases)				*P*-value for comparison of groups (additive model)	
										All subjects ($n = 745$)	Men only ($n = 635$)
		A	C	G	T	A	C	G	T		
rs2241712	-10807 (promoter)	0.64		0.36		0.71		0.29		0.01	0.04
rs1800469	-509 (promoter)		0.64		0.36		0.71		0.29	0.02	0.04
rs1982073	29 (exon 1)		0.44		0.56		0.36		0.64	0.001	0.02
rs6957	28344 (3′ genomic)	0.83		0.17		0.83		0.17		0.79	0.69
rs2241718	29343 (3′ genomic)		0.83		0.17		0.83		0.17	0.81	0.75

To assess for evidence of population stratification among the NAS and NETT participants, we genotyped a panel of 44 SNPs obtained through the SNP Consortium database. All of these SNPs were located in non-coding regions of genes that were not known to be associated with COPD phenotypes and were located on autosomal chromosomes. Genotyping of these SNPs was performed by unlabeled minisequencing reactions and mass spectrometry analysis as implemented in the SEQUENOM platform (Sequenom, San Diego, CA). Multiplex PCR and minisequencing assays were designed with SpectroDESIGNER software (Sequenom). Four- to seven-plex PCR reactions used 2.5 ng of genomic DNA in 5 ml volume. Protocol details and primer data are available at http://innateimmunity.net. Secondary multiplex single-primer minisequencing reactions were performed and analyzed with the Bruker Bi-flex MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA). Spectral output was analyzed using SpectroTYPER–RT software and by manual review.

All of the genotyping of the SNPs in the TGFB1 gene was performed by the TaqMan 5′ exonuclease assay using primers supplied by ABI. Major and minor allele probes were labeled with 5′ FAM and 5′ VIC fluorophore as reporters (ABI). Probe fluorescence signal detection employed the ABI Prism 7900 Sequence Detector System per manufacturer's specifications. Protocol details and primer data are available at http://www.innateimmunity.net. The average genotype completion rate for the five SNPs tested in the three populations studied was >97%.

Statistical analysis

Two-point and multipoint analysis of linkage between STR markers on chromosome 19 and quantitative COPD phenotypes in the Boston Early-Onset COPD Study was performed by a variance component method implemented in the SOLAR program (30). The quantitative phenotypes included in the linkage analysis were pre- and post-BD absolute volume spirometric measures (FEV_1 and FEV_1/FVC). Covariates in the variance component analysis included age, age^2, sex, height, height^2, pack-years of cigarette smoking and pack-years^2. Covariates that were significant at $P < 0.05$ in the polygenic model were retained in the linkage analysis model. Two-point and multipoint linkage analysis of qualitative COPD phenotypes was performed by the NPL All statistic implemented in the MERLIN program (31). Qualitative phenotypes included in the linkage analysis were pre- and post-BD mild to severe airflow obstruction ($FEV_1 < 80\%$ of predicted with $FEV_1/FVC < 90\%$ of predicted) and moderate to severe airflow obstruction ($FEV_1 < 60\%$ of predicted with $FEV_1/FVC < 90\%$ of predicted). All models were adjusted for age, sex, height and pack-years of cigarette smoking. Because the previously reported genome-wide linkage analysis for qualitative COPD phenotypes (4) was performed with the ALLEGRO program without covariates, we repeated that analysis using MERLIN for better comparability of the results obtained before and after the addition of flanking STR markers on chromosome 19. To examine a potential interaction between genes on chromosome 19 and cigarette smoking, we repeated the linkage analysis for qualitative and quantitative COPD phenotypes after excluding the phenotypic data of non-smokers from the analysis.

Among participants in the Boston Early-Onset COPD Study, the analysis of association between SNPs in TGFB1 and COPD phenotypes was performed by the extended pedigree family-based association test implemented in the PBAT program (32) under the assumption of either a dominant or additive mode of inheritance. To test for allelic association in a genomic region linked to COPD-related phenotypes, PBAT computed the marker distribution in the extended pedigrees using the empirical variance matrix estimator by Lake *et al.* (33). This association analysis was adjusted for age, sex, height and pack-years of cigarette smoking.

To assess whether there was evidence of population stratification among the NAS and NETT subjects, allelic association was first tested for each of the 44 SNPs using 2×2 contingency tables. Afterwards, an overall summary chi-square test statistic for the entire panel of SNPs was obtained by summing the individual chi-square test statistics (34).

The association between SNPs in TGFB1 and COPD among control subjects in the NAS and cases in the NETT was analyzed with the SAS Genetics program (SAS Institute, Cary, NC). Pairwise linkage disequilibrium (LD) between each pair of SNP loci was evaluated with a maximum likelihood method to infer phase for dual heterozygotes and was expressed as r^2. Among control subjects, Hardy–Weinberg equilibrium was tested at each SNP locus by a chi-square goodness-of-fit test. Association between each of the five SNPs and COPD was examined by 2×2 contingency tables, by Armitage's trend test to examine allelic additive effects,

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and by 2 × 3 contingency tables and the genotype case–control test to examine allelic dominant effects. Haplotype frequencies were estimated with an expectation-maximization algorithm in the SNPhap program (http://www-gene.cimr.cam.ac.uk/clayton/software/snphap.txt). Association between imputed haplotypes with frequency > 2% and COPD was tested for each haplotype and over all haplotypes with the haplo.score program (35).

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