Transforming Growth Factor- β Receptor-3 Is Associated with Pulmonary Emphysema

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Chronic obstructive pulmonary disease (COPD) is a heterogeneous syndrome, including emphysema and airway disease. Phenotypes defined on the basis of chest computed tomography (CT) may decrease disease heterogeneity and aid in the identification of candidate genes for COPD subtypes. To identify these genes, we performed genome-wide linkage analysis in extended pedigrees from the Boston Early-Onset COPD Study, stratified by emphysema status (defined by chest CT scans) of the probands, followed by genetic association analysis of positional candidate genes. A region on chromosome 1p showed strong evidence of linkage to lung function traits in families of emphysema-predominant probands in the stratified analysis (LOD score = 2.99 in families of emphysema-predominant probands versus 1.98 in all families). Association analysis in 949 individuals from 127 early-onset COPD pedigrees revealed association for COPD-related traits with an intronic single-nucleotide polymorphism (SNP) in transforming growth factor- β receptor-3 (TGFB3) ($P = 0.005$). This SNP was significantly associated with COPD affection status comparing 389 cases from the

CLINICAL RELEVANCE

In this article we have identified transforming growth factor- β receptor 3 as a novel candidate gene for chronic obstructive pulmonary disease (COPD) susceptibility, in a relevant COPD pathway. We also demonstrate how more precise phenotypic characterization may aid in the identification of genes for a heterogeneous complex disease.

National Emphysema Treatment Trial to 472 control smokers ($P = 0.04$), and with FEV₁ ($P = 0.004$) and CT emphysema ($P = 0.05$) in 3,117 subjects from the International COPD Genetics Network. Gene-level replication of association with lung function was seen in 427 patients with COPD from the Lung Health Study. In conclusion, stratified linkage analysis followed by association testing identified TGFB3 (betaglycan) as a potential susceptibility gene for COPD. Published human microarray and murine linkage studies have also demonstrated the importance of TGFB3 in emphysema and lung function, and our group and others have previously found association of COPD-related traits with TGF β 1, a ligand for TGFB3.

Keywords: betaglycan; chronic obstructive pulmonary disease; computed tomography; linkage; single nucleotide polymorphism

Genetic linkage and association studies have made significant strides in uncovering the genes for monogenic lung diseases, such as familial pulmonary hypertension (1) and autosomal dominant familial spontaneous pneumothorax (2). However, when these same techniques have been applied to common, complex respiratory disorders such as asthma and chronic obstructive pulmonary disease (COPD), the results have been less definitive; often the initial positive results were inconsistently replicated in follow-up studies (3–5). Multiple explanations have been proposed for the lack of replication, including small sample sizes, genotyping error, and population stratification (6). Differences in disease phenotypes between study populations may be a reason for nonreplication that is particularly relevant for COPD, a heterogeneous condition that includes both emphysema and airway disease.

Measurement of lung function using spirometry, though essential for the diagnosis of COPD (7), fails to distinguish between emphysema and airway disease. On the other hand, computed tomography (CT) scans of the chest can be used to confirm the presence and to grade the severity of emphysema. Therefore, we hypothesized that using chest CT scans to identify subjects with emphysema-predominant COPD would yield a more precise phenotype that would allow for the more efficient

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identification of candidate genes for COPD. We tested this hypothesis by performing stratified genome-wide linkage analysis in families of probands with CT-defined emphysema from the Boston Early-Onset COPD Study (8) to identify candidate genomic regions (Figure 1). Candidate genes from the linkage regions identified in the emphysema-stratified analysis were then tested for association with airflow obstruction in the Boston Early-Onset COPD Study families and in a case-control study comparing patients with emphysema from the National Emphysema Treatment Trial (NETT) (9) with community control subjects from the Normative Aging Study (NAS) (10). The results were replicated in the family-based International COPD Genetics Network (ICGN) study (11, 12) and in a cohort of patients with COPD from the Lung Health Study (LHS) (13). Results from this study have been previously presented as an abstract (14).

MATERIALS AND METHODS

Study Subjects

The Boston Early-Onset COPD Study enrolled extended pedigrees recruited through probands with physician-diagnosed COPD (age < 53 yr and FEV₁ < 40% predicted) (15). The linkage data set consisted of 585 individuals in 72 families (16); these individuals plus additional enrolled subjects constituted the association dataset of 949 individuals in 127 families (Table 1) (4). Phenotyping involved a questionnaire (17) and spirometry before and after inhaled bronchodilator (18). Chest CT scans

were not performed as part of the study protocol, but chest CTs were available from the clinical records of 44 of the probands in the linkage sample and 75 probands in the association sample. Chest CTs were scored by three to four readers, using a modification of the scoring system used in NETT (19, 20). Probands in the upper three quartiles of emphysema severity were defined as “emphysema-predominant” (8).

In the case-control study, emphysema cases were from NETT, a multicenter clinical trial of lung volume reduction surgery versus medical management for emphysema (9). Subjects had FEV₁ less than or equal to 45% predicted, hyperinflated lung volumes, and bilateral emphysema on chest CT scan (19). Participants in the NETT Genetics Ancillary Study provided a blood sample for genetic analysis. Control subjects with normal spirometry at the last study visit and with a smoking history of at least 10 pack-years (packs of cigarettes smoked per day multiplied by number of years smoking) were from the Normative Aging Study, a longitudinal cohort study of healthy male veterans in the Greater Boston area (10).

Subjects in the International COPD Genetics Network (ICGN) were recruited from ten centers in North America and Europe. Probands were aged 45 to 65 years, with post-bronchodilator FEV₁ less than 60% predicted, FEV₁/VC less than 90% predicted, a smoking history of at least 5 pack-years, and at least one sibling with a smoking history of 5 pack-years or more. Phenotype measurements included questionnaires, spirometry, and chest CT scans in a subset of the participants. Density mask analysis was used to quantify emphysema at a threshold of −950 HU; detailed chest CT methods have been described previously (11).

The Lung Health Study (LHS) was a 10-center randomized clinical trial, sponsored by the National Heart, Lung, and Blood Institute, to determine whether a program of smoking intervention and use of an inhaled bronchodilator could slow the rate of decline in pulmonary function in patients with COPD over a 5-year follow-up period (13). This analysis included 427 European American participants with mild-to-moderate COPD who were randomly selected from the LHS. At baseline, phenotyping involved questionnaires and spirometry before and after inhaled bronchodilator.

All studies were approved by the relevant institutional review boards, and study subjects provided written informed consent.

Linkage Analysis

A genome-wide panel of 377 autosomal short tandem repeat (STR) markers had been previously genotyped in the Boston Early-Onset COPD Study (16, 21). Linkage analyses for FEV₁ and FEV₁/FVC ratio (both pre- and post-bronchodilator) were performed in the 44 families with available proband chest CT scans and in the subset of 34 families with emphysema-predominant probands. Variance component linkage models were calculated in SOLAR (22), adjusted for age, sex, height, race, smoking status (ever versus never), pack-years of smoking, and quadratic terms where appropriate. Covariates significant at $P < 0.05$ were retained in the linkage models. Statistical significance for the subgroup analysis was determined by randomly permuting the emphysema-predominant proband status over 10,000 simulations.

Association Analysis

The initial association analyses were performed in the Boston Early-Onset COPD Study families and in the NETT-NAS case-control study. In TGFBR3, we initially genotyped six haplotype tagging (ht) single-nucleotide polymorphisms (SNPs), based on publicly available DNA sequence data from 23 European-American subjects in the Innate Immunity Program in Genomic Applications (IIPGA) (23); sequencing primers and results are available on the IIPGA website (www.innateimmunity.net). An additional 31 SNPs were genotyped to complete linkage disequilibrium (LD) tagging ($r^2 > 0.9$, minor allele frequency [MAF] ≥ 0.1), based on the sequencing data. In GCLM, we selected five LD-tagging SNPs based on data from white subjects (CEU) in the International HapMap Project (24). SNPs were genotyped using Taqman (Applied Biosystems, Foster City, CA) or Sequenom (San Diego, CA) platforms.

In the Boston Early-Onset COPD Study, SNPs were analyzed using the Family Based Association Test (25) implemented in PBAT software (26). Analyses of FEV₁ were adjusted for age, sex, height, smoking status, and pack-years, under additive genetic models, unless otherwise noted. SNPs were tested under the null hypothesis of link-

Figure 1. Study design incorporating stratified linkage analysis in the Boston Early-Onset COPD Study followed by genetic association studies in the Boston Early-Onset COPD Study, the National Emphysema Treatment Trial (NETT)–Normative Aging Study (NAS) case-control study, the International COPD Genetics Network, and the Lung Health Study.

TABLE 1. CHARACTERISTICS OF SUBJECTS IN THE BOSTON EARLY-ONSET COPD (EOCOPD) STUDY, THE NATIONAL EMPHYSEMA TREATMENT TRIAL (NETT) GENETICS ANCILLARY STUDY, THE INTERNATIONAL COPD GENETICS NETWORK (ICGN), AND THE LUNG HEALTH STUDY (LHS)

Characteristic	EOCOPD Probands	NETT	ICGN Probands	ICGN Relatives	LHS
Subjects	127	389	1,132	1,985	427
Age	48.1 ($\pm$ 4.7)	67.4 ($\pm$ 5.8)	58.3 ($\pm$ 5.4)	57.9 ($\pm$ 9.4)	48.7 ($\pm$ 6.9)
Sex					
Male	32 (25.2%)	250 (64.3%)	679 (60.0%)	1,025 (51.6%)	280 (65.5%)
Female	95 (74.8%)	139 (35.7%)	453 (40.0%)	960 (48.4%)	147 (34.5%)
Pack-years of smoking	38.9 ($\pm$ 21.9)	66.4 ($\pm$ 30.4)	52.2 ($\pm$ 29.1)	39.1 ($\pm$ 25.0)	40.3 ($\pm$ 19.1)
FEV ₁ % predicted	21.9 ($\pm$ 8.4)*	28.0 ($\pm$ 7.4)	36.1 ($\pm$ 12.9)*	83.1 ($\pm$ 26.1)*	79.2 ($\pm$ 6.2)
FEV ₁ /FVC Ratio	0.31 ($\pm$ 0.10)*	0.32 ($\pm$ 0.06)	0.37 ($\pm$ 0.12)*	0.64 ($\pm$ 0.14)*	0.65 ($\pm$ 0.06)
Subjects with chest CT scans	75	389	527	640	—
Emphysema score†	13.2 ($\pm$ 5.1)	16.3 ($\pm$ 4.0)	2.8 ($\pm$ 1.3)	1.3 ($\pm$ 1.1)	—
Subjects with quantitative CT analysis	—	358	409	578	—
CT percent emphysema, –950 Hounsfield Units	—	17.0 ($\pm$ 10.9)	26.3 ($\pm$ 14.8)	18.8 ($\pm$ 11.6)	—

Values are shown as N (%) or mean ($\pm$ SD)

* Subjects with post-bronchodilator (BD) values: EOCOPD $N = 118$; ICGN probands $N = 1,092$; ICGN relatives $N = 1,918$.

† EOCOPD and NETT: range, 0–24; ICGN: range, 0–5.

age, but no association. SNPs in the NETT-NAS case-control study were analyzed in SAS/Genetics (SAS Institute, Cary, NC), using the Armitage trend test (additive model), unless otherwise noted. LD was calculated in Haploview (27).

In the NETT-NAS case-control study, an additional panel of 195 common intergenic SNPs throughout the genome, excluding regions linked to COPD, was genotyped using the Illumina (San Diego, CA) GoldenGate assay in the NETT cases and in a majority of the NAS control subjects ($n = 409$). Based on this panel, there was no evidence of population stratification ($\chi^2_{195 \text{ df}} = 211.9$, $P = 0.19$) (28).

In the ICGN family-based study, the screening panel of 6 ht-SNPs in TGFB3 plus two additional SNPs that had been significant in both the Boston Early-Onset COPD Study and in NETT-NAS were genotyped using Sequenom assays. Data were analyzed using PBAT, under additive genetic models. Quantitative traits were adjusted for age, sex, pack-years of smoking, height (for spirometry), or weight (for CT densitometry measurement), and study center.

In the LHS, SNP selection and genotyping were conducted independently from the other three study samples. A total of 48 SNPs from TGFB3 were selected from the dbSNP database (<http://www.ncbi.nlm.nih.gov>). Criteria for SNP selection included the following, in order of priority: (1) regulatory and coding SNPs; (2) SNPs with known frequency, preferably MAF greater than or equal to 0.10 and no less than 0.05; (3) validated SNPs; (4) SNPs in intron/exon boundaries; and (5) SNPs in promoter and 3'UTR regions. SNPs were genotyped at the SNP Center at the Johns Hopkins Genetic Resources Core Facility (<http://snpcenter.grcf.jhmi.edu/>), with the Illumina BeadStation 500G GoldenGate genotyping platform. SNPs with MAF less than 0.01 ($n = 13$) were excluded from analyses. All SNPs were in Hardy-Weinberg equilibrium. The primary outcome was post-bronchodilator FEV₁, expressed as a percent of predicted (29). Linear regression models were used to adjust for potential confounders, including age, sex, and pack-years of smoking. Age and pack-years were significantly associated with FEV₁ (% predicted) in separate bivariate analyses ($P < 0.05$) and were included in the final model. The residuals from the linear regression model for FEV₁ (% predicted) adjusted for age and pack-years were then analyzed in an additive genetic model for each SNP. Analyses were performed with StataSE, version 8.0 (Stata Corp, College Station, TX) and PLINK (30).

RESULTS

Stratified Linkage Analysis

In the Boston Early-Onset COPD Study probands, chest CT scans were obtained for clinical indications and not as part of the study protocol. Since CTs were available for many of the probands but not their relatives, we could not perform stratified linkage analyses for CT emphysema scores. However, we were able to perform linkage analyses for spirometric measures of lung function (FEV₁ and FEV₁/FVC ratio, both before and after

bronchodilator administration) in families of 34 probands with emphysema-predominant COPD, defined on the basis of the proband CT scans. The stratified linkage analyses for lung function in emphysema-predominant proband families were considered surrogate phenotypes for emphysema. We compared the results of the stratified linkage analyses to the linkage analyses in the full set of 44 families in which the proband had a CT scan available. Chromosomal regions that showed increased evidence for linkage in the subgroup analysis were presumed to be linked to the emphysema subtype of COPD.

For FEV₁, a region on chromosome 1p showed stronger linkage evidence in the stratified analysis (Table 2 and Figure 2). For post-bronchodilator values of FEV₁, the LOD (logarithm of the odds of linkage) score of 1.98 in all families increased to 2.99 in the emphysema-predominant proband families; similar results were seen for pre-bronchodilator values. The increases in LOD scores were statistically significant ($P \leq 0.01$). A region on chromosome 8p also had an increase in LOD score in the stratified analysis of post-bronchodilator FEV₁. By genotyping additional STR markers on chromosome 8p, we had previously shown an attenuation of LOD score in this region, in an analysis that did not account for CT subtypes (31). The LOD score was also attenuated in the current analyses when we included the additional markers; therefore, we did not pursue further study of this chromosome 8p region.

For FEV₁/FVC ratio, chromosome 1p demonstrated a slight increase in LOD scores in the stratified analyses (see Table E1 in the online supplement). The most significant results in any of the linkage analyses were found for FEV₁/FVC on chromosome 2q, with higher LOD scores in all subjects than in the emphysema subgroup. We have previously demonstrated significant linkage for FEV₁/FVC on chromosome 2q (16, 32), and our group and others have found replicated association for SNPs in SERPINE2, a candidate gene located in this linked region (12, 33).

Genetic Association Analysis

Three potential candidate genes with relevance to COPD reported in the literature were located under the linkage peak on chromosome 1p (Figure 2). Several authors have shown the null deletion of glutathione S-transferase M1 (GSTM1) to be associated with COPD (34–36). In a previous article, we had found no association between the GSTM1 null variant and COPD in the NETT-NAS case-control sample (4); with this negative result, consistent replication would not have been possible, so we did not examine GSTM1 further in the present study. Two studies have examined variants in glutamate-cysteine ligase, modifier

TABLE 2. GENOME-WIDE LINKAGE ANALYSIS FOR FEV₁ IN THE BOSTON EARLY-ONSET COPD STUDY

Chromosome	cM	Pre-Bronchodilator		Post-Bronchodilator	
		Emphysema*	All†	Emphysema*	All†
1	138	2.89 (0.0006)	1.85 (0.0039)	2.99 (0.001)	1.98 (0.0044)
2	227	1.75 (0.006)	1.75 (0.0049)	1.50 (0.016)	1.53 (0.011)
3	207			1.45 (0.018)	1.57 (0.010)
6	89			2.17 (0.0047)	0.98 (0.040)
6	155			1.58 (0.014)	1.32 (0.019)
8	6	1.68 (0.0074)	1.68 (0.0058)	3.64 (0.0005)	3.09 (0.0005)
11	85			2.12 (0.0055)	1.11 (0.029)
14	135			1.78 (0.0097)	0.93 (0.045)

Chromosomal regions with LOD score > 1.5 (with empirical *P* values) in analyses of either families of emphysema-predominant probands or all families are shown.

* Significant covariates (*P* < 0.05) included age, sex, height, and pack-years of smoking.

† Significant covariates (*P* < 0.05) included age, sex, height, pack-years of smoking, and (pack-years)².

subunit (GCLM), though no association was found with COPD (37) or lung function decline (38). The gene for vascular cell adhesion molecule-1 (VCAM1) is also located in the region. However, this gene may be more relevant for airway disease than for emphysema (39), so we did not genotype VCAM1. We did examine transforming growth factor β receptor-3 (TGFBR3), given the prior associations between TGF-β1 variants and COPD (40, 41).

In the first stage, we genotyped six ht-SNPs in TGFBR3 and five LD-tagging SNPs in GCLM; subsequently, an additional 31 LD-tagging SNPs in TGFBR3 were typed (Figure 3). Over both stages, there were average genotype completion rates greater than 95% with very few pedigree inconsistencies in the family-based data. All SNPs were in Hardy-Weinberg equilibrium (*P* > 0.01) in the founder individuals from the Boston Early-Onset COPD Study families and in the NAS control subjects.

To improve statistical power, SNPs were tested for association in 949 individuals in 127 families from the Boston Early-Onset COPD Study rather than using only the families with proband CT scans. Results for the 6 ht-SNPs, plus two additional significant SNPs, in TGFBR3 in the Boston Early-Onset COPD Study, the NETT-NAS case-control study, and the ICGN family study are shown in Table 3. One intronic SNP, rs2296621, was significantly associated in all three cohorts (*P* = 0.005 in Boston Early-Onset COPD Study; *P* = 0.04 in NETT-NAS; *P* = 0.004 in ICGN), and another intronic SNP, rs2291477, was significantly associated in the NETT-NAS (*P* = 0.03) and ICGN (*P* = 0.01) studies and showed a trend for association in the Boston Early-Onset COPD Study (*P* = 0.06). These two SNPs are in LD with each other (*r*² = 0.91 in Boston Early-Onset COPD Study; *r*² = 0.87 in NETT-NAS and in ICGN).

In the ICGN study, these two SNPs were also significantly associated with an increased risk of severe COPD, defined by GOLD stages 3 or 4 (rs2296621, *P* = 0.001; rs2291477, *P* = 0.002), consistent with the increased odds ratios for COPD in the NETT-NAS case-control study. In the ICGN, the two SNPs showed trends for association with increased emphysema on quantitative image analysis of chest CT scans at −950 HU (rs2296621, *P* = 0.05; rs2291477, *P* = 0.06). Chest CT scans were available on a subset of NETT subjects, but the two TGFBR3 intronic SNPs and the Ser15Phe coding SNPs were not associated with percent emphysema at −950 HU in NETT. However, power was limited for this analysis, due to the reduced sample size of NETT subjects with quantitative CT scan data (Table 1).

Given the low minor allele frequency, the Ser15Phe (rs1805110) coding SNP in TGFBR3 was analyzed in dominant models, with significant associations for both pre-bronchodilator (*P* = 0.01) and post-bronchodilator FEV₁ (*P* = 0.02) in the Boston Early-Onset COPD Study and a significantly increased risk of COPD in the NETT-NAS case-control study (odds ratio, 1.51; 95% confidence interval, 1.04–2.20; *P* = 0.03). In the Boston Early-Onset COPD Study, TGFBR3 Ser15Phe was associated with GOLD stage 2 or greater COPD (*P* = 0.03) and was significantly associated with FEV₁ in an analysis restricted to 34 emphysema-predominant families (pre-bronchodilator, *P* = 0.03; post-bronchodilator, *P* = 0.045). However, Ser15Phe was not

Figure 2. Linkage analysis on chromosome 1 for forced expiratory volume in 1 s (FEV₁, post-bronchodilator) in all subjects (solid line) and in families of probands with emphysema-predominant COPD (dashed line) in the Boston Early-Onset COPD Study.

Figure 3. Association results for transforming growth factor- β receptor 3 single-nucleotide polymorphisms in the Boston Early-Onset COPD Study families (post-bronchodilator FEV₁, solid circles), the National Emphysema Treatment Trial (NETT)–Normative Aging Study (NAS) case-control study (open circles), the International COPD Genetics Network (ICGN) families (post-bronchodilator FEV₁, symbol “X”) and the Lung Health Study cohort (post-bronchodilator FEV₁ percent predicted, symbol “+”).

associated with FEV₁ or COPD in the ICGN study. Ser15Phe is not in LD with either of the two replicated intronic SNPs ($r^2 < 0.05$). Association results in the Boston Early-Onset COPD Study and in the NETT–NAS case-control study for the full set of 37 LD-tagging SNPs in TGFBR3 are shown in Table E2. Although several SNPs had P values < 0.05 in each cohort, no other SNPs showed replicated associations.

In the Lung Health Study (LHS), six SNPs in TGFBR3 were significantly associated with postbronchodilator values for FEV₁, expressed as a percent of predicted (Table 4). Neither rs2296621 nor rs2291477 nor any SNPs in tight LD ($r^2 > 0.8$) with these two SNPs were genotyped in the LHS. SNP rs1805110 (Ser15Phe) was not genotyped in the LHS, but two significant intronic SNPs, rs2634028 ($P = 0.02$) and rs2046737 ($P = 0.02$), are in complete LD ($r^2 = 1$) with rs1805110 in the white subjects (CEU) from the International HapMap Project (24). Another significantly asso-

ciated intronic SNP, rs2129972 ($P = 0.02$), is also in LD with rs1805110 in the HapMap ($r^2 = 0.81$).

A SNP 3' to the GCLM gene (rs2235970) was significantly associated with FEV₁ in the Boston Early-Onset COPD study, but no association with COPD susceptibility was seen in the NETT–NAS study (Table E3). No other SNPs in GCLM were significantly associated in either sample, and therefore were not followed up in the ICGN.

DISCUSSION

Using clinical chest CT scans, we have previously identified emphysema-predominant probands in the Boston Early-Onset COPD Study (8). Without CT scans on all family members, we could not perform genome-wide linkage analysis of CT emphysema; stratified linkage analysis of airflow obstruction pheno-

TABLE 3. ASSOCIATION ANALYSIS OF 8 SNPs (INCLUDING 6 HAPLOTYPE-TAGGING SNPs) IN TGFBR3 IN THE BOSTON EARLY-ONSET COPD STUDY (EOCOPD) FAMILIES, THE NATIONAL EMPHYSEMA TREATMENT TRIAL (NETT)–NORMATIVE AGING STUDY (NAS) CASE-CONTROL STUDY, AND THE INTERNATIONAL COPD GENETICS NETWORK (ICGN) FAMILY-BASED STUDY

SNP	Chrom. 1 Location*	Role	EOCOPD		NETT–NAS				ICGN	
			MAF	P Value FEV ₁ †	MAF Cases	MAF Controls	Odds Ratio (95% CI)	P (trend)	MAF	P Value FEV ₁ †
rs902	91920815	3' UTR	0.27	0.5	0.22	0.27	0.75 (0.60, 0.95)	0.02	0.24	0.6
rs2765889	91935931	Intron	0.23	1.0	0.19	0.25	0.73 (0.58, 0.93)	0.01	0.24	0.1
rs2296621	91936374	Intron	0.20	0.005	0.22	0.18	1.29 (1.01, 1.64)	0.04	0.19	0.004
rs1805113	91950526	Coding, synonymous	0.49	0.7	0.47	0.43	1.16 (0.95, 1.42)	0.2	0.45	0.002
rs2291477	91950923	Intron	0.22	0.06	0.24	0.19	1.30 (1.02, 1.64)	0.03	0.21	0.01
rs1805112	91958245	Coding, synonymous	0.40	0.046	0.42	0.39	1.15 (0.94, 1.42)	0.2	0.41	0.6
rs2306886	91972699	Intron	0.45	0.9	0.42	0.42	1.03 (0.84, 1.25)	0.8	0.45	0.3
rs1805110	92099633	Coding, Ser15Phe	0.065	0.02‡	0.095	0.069	1.51 (1.04, 2.20)‡	0.03‡	0.086	0.9‡

Definition of abbreviations: MAF, minor allele frequency; SNP, single-nucleotide polymorphism; TGFBR3, transforming growth factor- β receptor 3.

* March 2006 human genome reference sequence (NCBI Build 36).

† P value for association with post-bronchodilator FEV₁, using the Family-Based Association Test, implemented in PBAT software.

‡ Due to low minor allele frequency, rs1805110 was analyzed using a dominant model.

TABLE 4. ASSOCIATION ANALYSIS OF TGFBR3 SNPS AND POST-BRONCHODILATOR FEV₁ (% PREDICTED) IN THE LUNG HEALTH STUDY

SNP	Chrom. 1 Location*	β	P Value
rs12026758	91910754	1.9	0.2
rs2810894	91915822	-0.17	0.7
rs1804506	91920601	0.24	0.7
rs6604050	91926079	-0.30	0.5
rs2765887	91931035	0.11	0.8
rs2765889	91935931	-0.01	1.0
rs6660484	91945196	0.47	0.3
rs1805113	91950526	0.06	0.9
rs7532589	91965267	0.46	0.4
rs11578802	91970265	0.29	0.5
rs923378	91975471	-0.38	0.6
rs4658112	91981254	0.09	0.8
rs284170	91987216	1.3	0.03
rs7529421	91994013	-0.18	0.8
rs284176	91996564	0.49	0.3
rs12044500	92003913	-0.81	0.1
rs6700529	92008930	0.26	0.7
rs1473486	92014164	-0.32	0.4
rs4658270	92031370	-0.52	0.2
rs284165	92041549	-1.5	0.4
rs10493859	92046232	-0.59	0.2
rs10874996	92072672	1.0	0.04
rs2129972	92077838	-2.0	0.02
rs6686126	92083462	0.92	0.3
rs17575497	92088484	2.0	0.2
rs2634028	92093441	-2.0	0.02
rs2046737	92099224	-2.0	0.02
rs12727153	92104106	4.9	0.005
rs6656018	92108672	0.18	0.7
rs1192524	92113272	0.22	0.6
rs6680463	92130774	-0.43	0.3
rs12566180	92135390	-0.43	0.3
rs12141128	92146335	-0.42	0.3
rs2770186	92151431	-0.42	0.3
rs883873	92152890	-1.6	0.06

Definition of abbreviations: SNP, single-nucleotide polymorphism; TGFBR3, transforming growth factor- β receptor 3.

* March 2006 human genome reference sequence (NCBI Build 36).

SNP associations with $P < 0.05$ are shown in boldface type.

types in families of emphysema-predominant probands served as a surrogate phenotype. We identified a region on chromosome 1p with significantly increased evidence for linkage in the subset of families of the emphysema-predominant probands. This linkage peak from the stratified analysis pointed to a chromosomal region that may be important in emphysema, in addition to the linkage peaks for spirometric traits previously identified in all subjects (16). In a previous publication, we found no association with GSTM1, a potential candidate gene (4). In the current study, we have tested two additional candidate genes for emphysema located in the linked region, finding SNP-level replication of the association between two intronic SNPs in TGFBR3 (also known as betaglycan) and COPD phenotypes in three study samples (Boston Early-Onset COPD Study, NETT-NAS and ICGN), with gene-level replication in a fourth study (LHS). A coding SNP (Ser15Phe), or proxy SNPs for this coding SNP, was associated in three of the four populations. In the Boston Early-Onset COPD Study, the ICGN, and the LHS, the main associated phenotype was FEV₁; subjects in NETT-NAS were ascertained based on FEV₁ levels, among other criteria. The two intronic SNPs also showed a trend toward association with quantitative emphysema measurements in the subset of ICGN participants who had chest CT scans. In NETT, all subjects had emphysema on chest CT, and in the Boston Early-Onset COPD Study, the association remained significant in the families of emphysema-predominant probands,

also pointing to the potential relevance of TGFBR3 as a candidate gene for the emphysema component of COPD. Chest CT scans were not available in the Lung Health Study.

The only reported genome-wide linkage analyses for COPD-related traits have been performed in the Boston Early-Onset COPD Study (16, 21). However, a genome-wide linkage analysis in 144 families from the Collaborative Study for the Genetics of Asthma found evidence of linkage to asthma on chromosome 1p only in subjects exposed to environmental tobacco smoke (42), pointing to the potential relevance of genes on chromosome 1p in smoking-related lung diseases. Our study is the first published report of an association between polymorphisms in TGFBR3 and COPD.

Other human and murine genetics studies demonstrate the potential importance of TGFBR3 in COPD and emphysema. In a microarray study of human lung tissue, Golpon and colleagues found reduced expression of TGFBR3 in lungs from five patients with emphysema compared with five normal lungs (43); this difference was validated by RT-PCR. In a genome-wide linkage analysis in mice, Reinhard and coworkers found significant linkage to lung function on murine chromosome 5, where the orthologous murine gene *tgfb3* is located (44). Sequencing of the *tgfb3* gene found three nonsynonymous coding variants between the two parental strains. However, Reinhard and colleagues did not describe a SNP corresponding to the human Ser15Phe variant, and a similar mouse variant is not found in the dbSNP database.

The functional effects of the two associated intronic SNPs are unknown. These SNPs may have functional consequences, or more likely, they are in LD with the causal variant(s). The Ser15Phe variant is located in the signal peptide of TGFBR3; the functional consequence of this amino acid change is not known, but it is predicted to be possibly damaging, according to PolyPhen analysis (45). We demonstrated associations with several SNPs in this large gene (223.5 kb), suggesting that multiple TGFBR3 variants may affect emphysema susceptibility. It also possible that the causal variant or variants are not located in the TGFBR3 gene, and that the associations we found are due to LD with other chromosome 1 genes. However, we did not find associations with two other COPD candidate genes in the region, and TGFBR3 is known to be expressed in human emphysematous lung (43). Several other lines of evidence have pointed to the importance of the TGF- β pathway in COPD (40, 46, 47).

The linkage and association analyses in the Boston Early-Onset COPD Study are limited by the availability of chest CT scans only on the probands; since CT scans were performed for clinical indications at the discretion of the local physician, and were not part of the study protocol, CTs were not available in the family members. Linkage and association analyses for qualitative or quantitative CT traits could not be performed, though we have previously demonstrated that emphysema predominance on CT can be used to define clinically relevant subgroups (8). We did not find association with emphysema measurements in NETT, though the sample size in NETT with quantitative CT data was limited. In the larger sample from the ICGN study, we were able to find trends for association with quantitative emphysema measurements.

Spurious association due to multiple testing is a concern in genetic association studies (6). To guard against multiple testing, we used a staged genotyping and replication approach (Figure 1). First, we screened a limited number of SNPs in TGFBR3 in the Boston Early-Onset COPD Study families and in the NETT-NAS case-control study. Only after identifying a replicated association did we test additional SNPs in that gene. The screening set of ht-SNPs plus additional significant SNPs were then tested in the large ICGN family study. We then compared the results to independent genotyping performed in a separate cohort study

of patients with COPD from the LHS. Consistent replication of the results across multiple patient samples, using different study designs, offers strong protection against false positive results (5). The use of family-based study designs in the Boston Early-Onset COPD Study and the ICGN and the testing of a panel of unlinked SNPs in the NETT-NAS case-control study protect against spurious association due to population stratification.

To refine the heterogeneous syndrome of COPD, we used chest CT scans to define a set of early-onset COPD probands with emphysema-predominant COPD. Genome-wide linkage analysis followed by association testing identified variants in *TGFBR3*, a biologically plausible candidate gene, to be associated with COPD and with FEV₁, an important intermediate phenotype. SNP-level replication was confirmed in follow-up studies comparing emphysema cases from NETT with control smokers and in a large family-based study from the ICGN. The same SNPs were not tested in the LHS, but gene-level replication was found for *TGFBR3*. COPD is, by definition, a complex disease. There are likely to be some genes relevant for COPD in general and other genes relevant only for COPD subtypes, such as emphysema or airway disease. Genetic studies in patients with COPD defined only on the basis of airflow obstruction may be sufficient to identify the former set of genes. However, more precise characterization of subjects with COPD, using chest CT scans, will be required to find distinct genes for emphysema and airway disease.

Conflict of Interest Statement: S.G.P. is a full time employee of GlaxoSmithKline (GSK). H.O.C. received \$11,000 in 2005 and \$4,800 in 2006 and 2007 for serving on an advisory board for GSK. In addition, H.O.C. is the co-investigator on two multi-center studies sponsored by GSK and has received travel expenses to attend meetings related to the project. H.O.C. has three contract service agreements with GSK to quantify the CT scans in subjects with COPD and a service agreement with Spiration, Inc. to measure changes in lung volume in subjects with severe emphysema. A percentage of H.O.C.'s salary between 2003 and 2006 (\$15,000/year) derives from contract funds provided to a colleague (Peter D. Pare) by GSK for the development of validated methods to measure emphysema and airway disease using computed tomography. H.O.C. is the co-investigator (principal investigator: D. Sin) on a Canadian Institute of Health-Industry (Wyeth) partnership grant. There is no financial relationship between any industry and the current study. E.K.S. received an honorarium for a talk on COPD genetics in 2006, and grant support and consulting fees from GSK for two studies of COPD genetics. E.K.S. received an honorarium from Wyeth for a talk on COPD genetics in 2004. E.K.S. received an honorarium from Bayer for a symposium at the ERS Meeting in 2005. E.K.S. received an honorarium for a talk at the Lund Symposium in 2007 and consulting fees in 2008 from AstraZeneca. None of the other authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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