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## Genetic Modifiers of Lung Disease in Cystic Fibrosis

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### ABSTRACT

#### BACKGROUND

Polymorphisms in genes other than the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene may modify the severity of pulmonary disease in patients with cystic fibrosis.

#### METHODS

We performed two studies with different patient samples. We first tested 808 patients who were homozygous for the  $\Delta F508$  mutation and were classified as having either severe or mild lung disease, as defined by the lowest or highest quartile of forced expiratory volume in one second ( $FEV_1$ ), respectively, for age. We genotyped 16 polymorphisms in 10 genes reported by others as modifiers of disease severity in cystic fibrosis and tested for an association in patients with severe disease (263 patients) or mild disease (545). In the replication (second) study, we tested 498 patients, with various *CFTR* genotypes and a range of  $FEV_1$  values, for an association of the *TGF $\beta$ 1* codon 10 CC genotype with low  $FEV_1$ .

#### RESULTS

In the initial study, significant allelic and genotypic associations with phenotype were seen only for *TGF $\beta$ 1* (the gene encoding transforming growth factor  $\beta$ 1), particularly the -509 and codon 10 polymorphisms (with P values obtained with the use of Fisher's exact test and logistic regression ranging from 0.006 to 0.0002). The odds ratio was about 2.2 for the highest-risk *TGF $\beta$ 1* genotype (codon 10 CC) in association with the phenotype for severe lung disease. The replication study confirmed the association of the *TGF $\beta$ 1* codon 10 CC genotype with more severe lung disease in comparisons with the use of dichotomized  $FEV_1$  for severity status ( $P=0.0002$ ) and  $FEV_1$  values directly ( $P=0.02$ ).

#### CONCLUSIONS

Genetic variation in the 5' end of *TGF $\beta$ 1* or a nearby upstream region modifies disease severity in cystic fibrosis.

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**C**YSTIC FIBROSIS IS A RECESSIVE GENETIC disorder that reflects mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene.<sup>1</sup> Classic cystic fibrosis reflects two loss-of-function alleles, whereas non-classic cystic fibrosis is characterized by at least one mutant *CFTR* allele that confers partial function and, in most cases, better survival.

There is great variability of pulmonary phenotype and survival in cystic fibrosis, even among patients who are homozygous for the most prevalent mutation,  $\Delta F508$ .<sup>1,2</sup> Although environmental influences may modify clinical disease, there is probably additional genetic variation (i.e., the presence of “modifier” genes<sup>3</sup>) that contributes to the expression of the final phenotype.

To assess the effect of non-*CFTR* genetic polymorphisms on the clinical phenotype, we studied variants of 10 genes previously reported as modifiers in cystic fibrosis, including genes encoding  $\alpha 1$ -antitrypsin (*\alpha 1AP*)<sup>4-8</sup>; angiotensin-converting enzyme (*ACE*)<sup>9</sup>;  $\beta_2$ -adrenergic receptor (*ADR\beta\_2*)<sup>10</sup>; two glutathione S-transferases (*GSTM1* and *GSTP1*)<sup>11-13</sup>; interleukin-10 (*IL10*)<sup>9</sup>; mannose-binding lectin 2 (*MBL2*)<sup>14-18</sup>; nitric oxide synthase 3 (*NOS3*)<sup>19</sup>; transforming growth factor  $\beta 1$  (*TGF\beta 1*)<sup>9,20</sup>; and tumor necrosis factor  $\alpha$  (*TNF\alpha*).<sup>9,12</sup> Chloride conductance that is linked to genetic background may modulate the clinical phenotype in cystic fibrosis, but the specific loci and genes have not been identified.<sup>21</sup>

In the initial study, we tested patients who were homozygous for the  $\Delta F508$  mutation and had one of the two extremes of phenotypes associated with lung function (i.e., severe or mild impairment) on the basis of the hypothesis that adverse and beneficial genetic variants would be enriched, respectively, in these two groups of disease severity. The classification of pulmonary function as severe or mild (i.e., the lowest or highest quartile of lung function for age) was confirmed by estimating final forced expiratory volume in one second ( $FEV_1$ ) for each patient on the basis of multiple spirometric measurements during the five years before enrollment (see the Supplementary Appendix, available with the full text of this article at [www.nejm.org](http://www.nejm.org)). Genotypes of the modifier variants were compared in the groups of patients with the severe or mild phenotype, in a manner similar to a case-control design. We replicated our findings in a different population of patients with cystic fibrosis.

## METHODS

### PATIENTS

For the initial study, the sample consisted of patients with cystic fibrosis, enrolled from 44 sites, who were homozygous for the  $\Delta F508$  mutation. The 840 patients who were initially enrolled were chosen because their  $FEV_1$  measurements were in the lowest quartile or highest quartile for age among  $\Delta F508$  homozygotes. The lung function in 275 of these patients was classified as severely impaired (lowest quartile), and that of 565 patients as mildly impaired (highest quartile). A total of 32 patients were excluded because they had inadequate spirometry (2 patients), were not homozygous for the  $\Delta F508$  mutation (8), or did not have more than a 90 percent probability of having lung function congruent with that of others in the severe or mild category (22) (see Validation of Subjects in the Supplementary Appendix). There were 808 patients in the final data set. No patient was excluded because of race or ethnic background; 96.7 percent of the patients were self-identified as white.

In the replication study, we tested 498 patients whose sputum cultures were negative for *Burkholderia cepacia* and who had *CFTR* genotypes associated with pancreatic exocrine insufficiency; 70.5 percent were  $\Delta F508$  homozygotes. As in the initial study, no patient was excluded because of race or ethnic background. The majority of the patients in the replication study were from a research study at the University of North Carolina at Chapel Hill, Case Western Reserve University in Cleveland, or the Hospital for Sick Children in Toronto (Supplementary Appendix). The study was approved by the biomedical institutional review board of the University of North Carolina and the institutional review board of each participating institution. Patients and parents of minors provided written informed consent.

### DATA COLLECTION

For the initial study, each patient received a unique code that was used to allow data processing while maintaining anonymity. Key data were obtained from source documents, including pulmonary-function reports from the previous five years, which provided measurements of height and weight to calculate body-mass index as an index of nutrition, and sputum microbiologic testing during the previous three years. Other data were obtained on case-

report forms. For the replication study, we used FEV<sub>1</sub> data that were available at the sites (Supplementary Appendix).

#### GENOTYPING

For the initial study, genetic testing was performed by sequencing  $\alpha 1AP$ -Z and *MBL2* B, C, and D “null” alleles; by single-nucleotide polymorphism (SNP) technology (BeadArray, Illumina) for  $\alpha 1AP$ -S and *G1237A* alleles, *GSTP1*, *IL10*, *MBL2* promoter variants X and Y, *NOS3*, and *TGF $\beta$ 1*; or by published methods for *ACE*, *ADR $\beta$ 2*, *GSTM1*, and *TNF $\alpha$*  (Supplementary Appendix). *MBL2* structural (null) variants (B, C, and D) were combined to construct the O/O genotype. Other possible modifier genes for lung disease and their SNPs (65 and 135, respectively) were genotyped by Illumina but were not examined for the initial study. At least 798 patients were successfully genotyped for most genetic variants, except for four alleles:  $\alpha 1AP$ -Z (781 patients), two *ADR $\beta$ 2* alleles (741 and 743 patients, respectively), and *TNF $\alpha$*  (743 patients). For regions flanking *TGF $\beta$ 1*, we tested 31 SNPs at the Genome Analysis Facility at the University of North Carolina at Chapel Hill (TaqMan SNP Genotyping Assay by ABI-7900HT, Applied Biosystems) (Supplementary Appendix). After correction for multiple testing, none of the SNPs in the initial study showed significant overall departure from Hardy–Weinberg equilibrium.<sup>22</sup> For the replication study, *TGF $\beta$ 1* codon 10 genotypes were determined by sequencing (at the University of North Carolina and Case Western Reserve University) and by allele-specific oligonucleotide testing (at the Hospital for Sick Children) (Supplementary Appendix).

#### STATISTICAL ANALYSIS

For the initial study, the association between polymorphisms and the phenotype for the severity of impairment of lung function was assessed with the use of Fisher’s exact tests of genotype and allele frequencies. All tests were two-sided, with an alpha level of 0.05 considered to indicate statistical significance. Unadjusted P values are reported, whereas multiple-comparison corrections were performed for the 16 polymorphisms by recomputing the tests for 10,000 random permutations of severity-of-impairment status. The adjusted P value was based on the permutation distribution of the smallest P value among the 16.

For *TGF $\beta$ 1* polymorphisms, estimated haplotype reconstructions<sup>23</sup> were compared with severity status to compute standard contingency table (chi-square) statistics. P values for these haplotypes were assessed with the use of 10,000 random permutations of severity status. Logistic regression was used to estimate the effect of *TGF $\beta$ 1* genotypes on the odds of a patient’s having the phenotype for severe impairment under three genetic models (recessive, codominant, and dominant). Additional multivariate logistic-regression analyses included the covariates of the presence or absence of diabetes mellitus, *Pseudomonas aeruginosa* airway infection, meconium ileus, and a physician’s diagnosis of asthma.<sup>24</sup> Linkage disequilibrium patterns among *TGF $\beta$ 1* polymorphisms and 31 flanking SNPs were analyzed with the use of E-M estimation of haplotype frequencies, as implemented with the LDMAX program in the GOLD software package.<sup>25,26</sup>

For the replication study, we used results from the initial study to guide the design and to establish primary and secondary analytic approaches (Supplementary Appendix). Analysis of the initial study data suggested that the use of a dichotomized phenotype and a recessive model for an increased risk of severity of impairment due to the codon 10 C allele would provide the greatest power. The primary analysis for the replication study was performed with Fisher’s exact test of association between the *TGF $\beta$ 1* codon 10 CC genotype and FEV<sub>1</sub> status below or above a defined threshold (an FEV<sub>1</sub> of 68 percent of the predicted value, estimated for the age of 20 years), with the use of a mixed linear regression model (Supplementary Appendix). In the secondary analysis in the replication study, we used a Wilcoxon test to compare FEV<sub>1</sub> values for patients with the codon 10 CC genotype with those for patients with other (TC/TT) genotypes (Supplementary Appendix). As an additional conservative approach, two-sided P values were calculated for both primary and secondary analyses of the replication data. To reduce multiple comparisons, association tests were performed only on the entire sample in the replication study. Tests of Hardy–Weinberg equilibrium were also performed within groups that were classified according to the severity of impairment of lung function,<sup>22</sup> following predictions that departures from Hardy–Weinberg equilibrium can arise with associated genes in case-control studies<sup>27</sup> (Supplementary Appendix).

RESULTS

INITIAL STUDY

Characteristics of Patients

The pulmonary and nutritional characteristics of 263 patients with the phenotype for severe impairment of lung function were distinctly different from those of 545 patients with the phenotype for mild impairment (Table 1). Even though patients in the group with the mild phenotype were approximately 12 years older than patients in the group with the severe phenotype, the average FEV<sub>1</sub> at enrollment of patients with mild impairment was higher and the yearly decline in FEV<sub>1</sub> was approximately one third of that in patients with severe impairment. The group with the mild phenotype had relatively preserved nutrition (on the basis of the body-mass index), whereas the group with the severe phenotype was malnourished. The two groups were sim-

ilar with respect to the sex ratio; the presence or absence of *P. aeruginosa* in sputum; diabetes mellitus (when adjusted for age) or asthma; and enrollment site (86.4 percent of patients with the severe phenotype were matched by enrollment of one or more patients with the mild phenotype from the same site). When we divided the mild group into two age groups according to predetermined criteria (Supplementary Appendix), there were even more striking differences between the severe group and the younger mild group with respect to FEV<sub>1</sub> and its rate of decline. The patients in the older mild group had FEV<sub>1</sub> values similar to those of the severe group, despite being approximately 22 years older.

Genotype and Allelic Associations

In the analysis of previously reported genes (Table 2), significant associations with phenotype were

**Table 1. Characteristics of 808 Patients with Cystic Fibrosis Homozygous for the ΔF508 Mutation, According to the Phenotype for Severe or Mild Impairment of Lung Function and Age (Initial Study).\***

Variable	Degree of Impairment		P Value†	Patients with Mild Impairment, Divided into Two Groups by Age	
	Severe‡ (N=263)	Mild§ (N=545)		Younger (N=299)	Older (N=246)
Age (yr)					
Range	8–25	15–55		15–28	29–55
Mean	16.2±4.1	28.6±9.7	<0.001	20.9±4.0	38.0±5.3
Sex (% male)	49.4	55.6	0.10	50.8	61.4
FEV <sub>1</sub> (% of predicted value)¶	46.6±16.1	72.4±28.1	<0.001	90.8±16.2	50.0±22.9
FEV <sub>1</sub> decline (%/yr)¶	3.65±2.20	1.35±1.51	<0.001	1.10±1.77	1.64±1.04
Median predicted survival (age in yr)‖	31.4	56.6	<0.001	58.0	55.3
Body-mass index (percentile)**	19.6±21.7	44.0±26.1	<0.001	47.0±24.1	40.4±28.0
Positive test for <i>P. aeruginosa</i> (%)††	89.0	86.1	0.25	84.0	88.6
Diabetes mellitus (%)‡‡	15.6	24.0	0.006	14.1	36.2
Asthma (%)§§	19.4	22.0	0.39	19.7	24.8

\* Plus-minus values are means ±SD.

† P values were calculated with the Wilcoxon rank-sum test for continuous variables and the chi-square test for categorical variables.

‡ Severe impairment was defined as being in the lowest 25th percentile of forced expiratory volume in one second (FEV<sub>1</sub>) for age, as compared with patients of the same age who were homozygous for the ΔF508 mutation.

§ Mild impairment was defined as being in the highest 25th percentile of FEV<sub>1</sub> and survival (for older patients), as compared with ΔF508 homozygotes the same age.

¶ Values reported are means and standard deviations of empirical Bayes estimates for individual patients, obtained by fitting a mixed model to data from all 808 patients. The FEV<sub>1</sub> percentage of predicted value is at the time of enrollment.

‖ The predicted survival was calculated from a combination of the patient's age and best yearly FEV<sub>1</sub> percentage of predicted value with the use of estimates derived from a joint model of lung function and survival.<sup>28</sup>

\*\*The body-mass index was referenced to age- and sex-matched normal persons, of whom the 50th percentile was used as the median value.

††The presence of infection was determined by sputum culture.

‡‡The presence of diabetes was determined on the basis of an abnormal fasting glucose level, an abnormal result on a glucose-tolerance test, or the use of oral hypoglycemic agents or insulin.

§§ The presence of asthma was determined on the basis of criteria of the American Thoracic Society.

**Table 2. Prevalence of Polymorphic Genotypes According to the Severe Phenotype (N=263) or the Mild Phenotype (N=545) (Initial Study).<sup>\*,†</sup>**

Gene and Reference <sup>‡</sup>	Variant <sup>‡</sup>	Reference SNP	Impairment of Lung Function	Patients with the Severe Phenotype (N=263)		Patients with the Mild Phenotype (N=545)		P Value <sup>§</sup>			
				Genotype	%	Genotype	%				
$\alpha$ 1AP <sup>4,8</sup>	S allele (T2313A)	17580	Severe	AA	91.5	AT	8.5	TT	0	260	1.00
	Z allele (G4627A)	None	Mild	AA	91.4	AT	8.5	TT	0.2	544	0.39
3' enhancer (G1237A)		11568814	Mild	GG	96.0	GA	4.0	AA	0	252	0.75
			Severe	GG	97.2	GA	2.8	AA	0	529	0.32
ACE <sup>9</sup>	D or I deletion	NA	Mild	GG	85.8	GA	13.5	AA	0.8	260	0.62
			Severe	GG	84.7	GA	14.7	AA	0.6	544	0.91
ADRB2 <sup>10</sup>	(A46G)	1042713	Mild	DD	39.3	DI	39.3	II	21.4	262	0.0008
	(C79G)	1042714	Severe	DD	38.2	DI	44.7	II	17.1	544	0.06
GSTM1 <sup>11,13</sup>	Null deletion	NA	Mild	GG	39.1	GA	48.5	AA	12.3	235	0.45
			Severe	GG	41.7	GA	48.0	AA	10.3	506	0.50
GSTP1 <sup>13</sup>	(A1375G)	947894	Mild	CC	26.8	CG	56.6	GG	16.6	235	0.81
	(G-1082A)	1800896	Severe	CC	29.9	CG	51.6	GG	18.5	508	0.96
MBL2 <sup>14,18</sup>	O <sup>¶</sup>	NA	Mild	CC	52.1	—	—	DN/NN	47.9	261	0.58
	XA/O <sup>  </sup>	NA	Severe	DD	54.9	—	—	DN/NN	45.1	539	1.00
NOS3 <sup>19</sup>	T5220G	1799983	Mild	AA	46.2	AG	44.2	GG	9.6	260	0.54
	Promoter (C-509T)	1800469	Severe	AA	44.0	AG	45.1	GG	10.9	543	0.006
TGFB1 <sup>9,20</sup> **	Codon 10 (C29T) <sup>††</sup>	1982073	Mild	GG	23.1	GA	55.4	AA	21.5	260	0.06
	Codon 25 (G74C)	1800471	Severe	GG	24.1	GA	55.0	AA	21.0	544	0.91
TNF $\alpha$ <sup>9,12</sup>	Promoter (G-308A)	1800629	Mild	AA	59.5	AO	35.5	OO	5.0	262	0.06
			Severe	AA	58.2	AO	37.5	OO	4.3	536	0.0008
			Mild	Other	85.8	XA/O	9.2	O/O	5.0	262	0.54
			Severe	Other	85.6	XA/O	10.0	O/O	4.3	536	0.006

\* NA denotes not applicable.  
<sup>†</sup> GenBank accession numbers for these genes are  $\alpha$ -AP, NT\_026437; ACE, NT\_010783; ADRB2, NT\_029289; GSTM1, NT\_019273; GSTP1, NT\_033903; IL10, NT\_021877; MBL2, NT\_008583; NOS3, NT\_007914; TGFB1, NT\_011109; and TNF $\alpha$ , NT\_007592.  
<sup>‡</sup> Genetic variants of genes were previously studied as modifiers in cystic fibrosis. Variants are numbered from start of translation, except as otherwise noted.  
<sup>§</sup> All P values were calculated with the use of Fisher's exact test of comparisons of three genotypes with the degree of impairment of lung function, unless otherwise specified.  
<sup>¶</sup> O denotes any of the "null" structural polymorphisms (B, C, or D), and A is the normal structural allele. P values are based on comparisons of the O/O ("null") genotype with the remaining genotypes.  
<sup>||</sup> XA is the low-expression promoter variant (X) coupled to the normal structural (A) sequence. P values are based on comparisons of the O/O or XA/O genotype with the remaining genotypes.  
<sup>\*\*</sup> P=0.01 for the TGFB1 codon 10 variant after multiple-comparison correction, and P=0.007 for permutation-based test of the haplotype of 3 SNPs in TGFB1.  
<sup>††</sup> Variant nucleotides are numbered from the start of transcription.

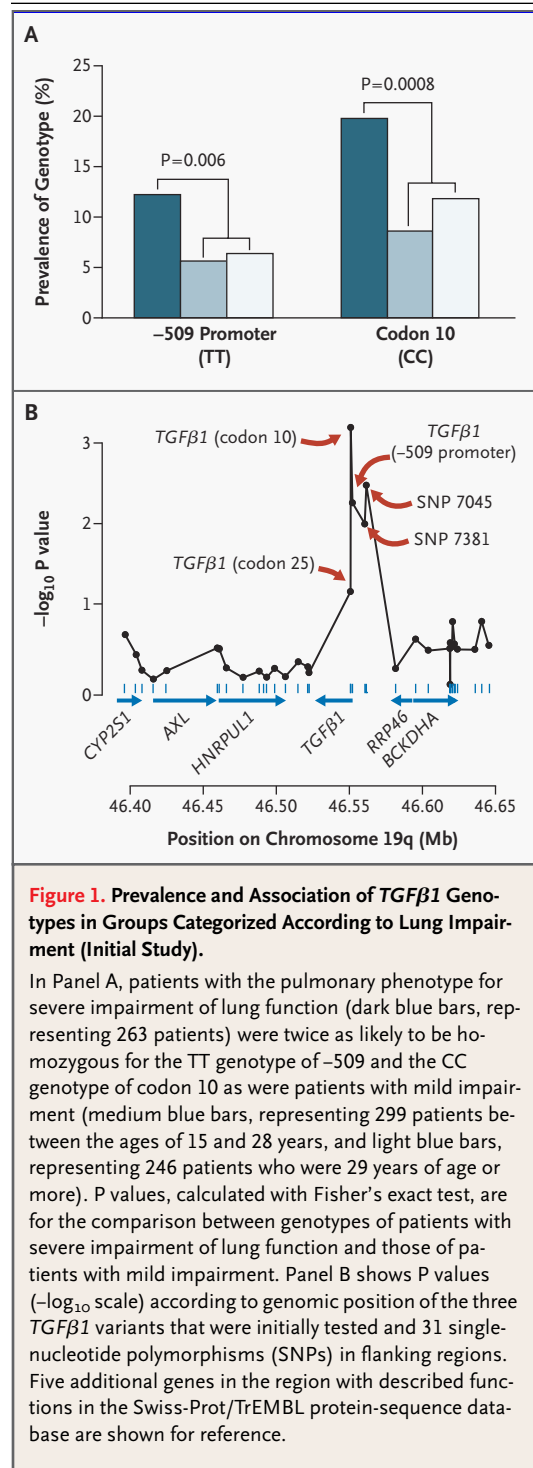
seen only for *TGFβ1* variants; the codon 10 variant had a multiple-comparison corrected P value of 0.01 (the most significant association among the 16 in Table 2). The frequencies of the genotypes and “minor” alleles for genetic variants tested were similar to those previously reported among white subjects (Table 2).<sup>4-15,19,20,29-32</sup> In further analyses of subgroups, there were four significant or suggestive P values (between 0.01 and 0.10) for variants *α1AP-Z* and *GSTM1* (Table 1 in the Supplementary Appendix); none of the P values were significant after multiple-testing corrections.

The *TGFβ1* variant genotypes were strongly associated with phenotype (Table 2). A statistical association was also seen in a permutation test of reconstructed haplotype frequencies for the three *TGFβ1* SNPs ( $P=0.007$ ). In tests of direct allelic association, only two *TGFβ1* SNPs (–509 and codon 10) were significant ( $P=0.009$  and  $P=0.001$ , respectively). The prevalence of two *TGFβ1* genotypes in the severe and mild groups is shown in Figure 1. Patients with the phenotype for severe impairment of lung function were twice as likely to be homozygous for the TT genotype of –509 and the CC genotype of codon 10.

Further analyses were undertaken with the use of logistic regression for the two most significant *TGFβ1* variants, with the use of three genetic models (recessive, codominant, or dominant) to test for the effect of the higher-risk genotype on the odds of having the severe phenotype (Table 3). The recessive and codominant models were most highly significant; for the recessive model, the odds ratios for genotype effects were 2.18 and 2.25 for the overall analysis, and they ranged from 1.58 to 3.16 for subgroup analyses by sex. Similar results were obtained after adjustment for four covariates: the presence or absence of diabetes mellitus, meconium ileus at birth, *P. aeruginosa* infection, and asthma (Table 2 in the Supplementary Appendix).

#### SNPs Flanking *TGFβ1* and Linkage Disequilibrium Patterns

We reasoned that testing additional SNPs around *TGFβ1* would further elucidate the genetic association and linkage disequilibrium patterns (Fig. 1 in the Supplementary Appendix). A total of 31 SNPs surrounding *TGFβ1* were genotyped; Figure 1 displays the P values for the association of SNP genotypes with the severity of disease ( $-\log_{10}$  scale), shown according to genomic position. The *TGFβ1* variant codon 10 retained the strongest evidence



**Figure 1. Prevalence and Association of *TGFβ1* Genotypes in Groups Categorized According to Lung Impairment (Initial Study).**

In Panel A, patients with the pulmonary phenotype for severe impairment of lung function (dark blue bars, representing 263 patients) were twice as likely to be homozygous for the TT genotype of –509 and the CC genotype of codon 10 as were patients with mild impairment (medium blue bars, representing 299 patients between the ages of 15 and 28 years, and light blue bars, representing 246 patients who were 29 years of age or more). P values, calculated with Fisher’s exact test, are for the comparison between genotypes of patients with severe impairment of lung function and those of patients with mild impairment. Panel B shows P values ( $-\log_{10}$  scale) according to genomic position of the three *TGFβ1* variants that were initially tested and 31 single-nucleotide polymorphisms (SNPs) in flanking regions. Five additional genes in the region with described functions in the Swiss-Prot/TrEMBL protein-sequence database are shown for reference.

of an association with severity. Of the flanking SNPs, only the two directly 5’ of *TGFβ1* (7381 and 7045, GenBank accession number NT\_011109) were significant, and those SNPs were in strong linkage

**Table 3. Logistic-Regression Analysis of *TGFβ1* Genetic Variants Associated with Severe Lung Disease among Patients with Severe Impairment (N=260) and Mild Impairment (N=544) (Initial Study).\***

Genetic Variant and Reference SNP	Recessive Model		Codominant Model		Dominant Model	
	Odds Ratio (95% CI)	P Value	Odds Ratio (95% CI)	P Value	Odds Ratio (95% CI)	P Value
All patients (N=804)						
-509 T (1800469)	2.25 (1.34–3.76)	0.002†	1.90 (1.19–3.01)	0.007‡	1.30 (0.97–1.75)	0.08§
Codon 10 C (1982073)	2.18 (1.44–3.29)	0.0002¶	2.10 (1.36–3.26)	0.0009	1.37 (1.01–1.87)	0.04**
Male patients (N=430)						
-509 T (1800469)	2.25 (1.11–4.58)	0.02	1.97 (1.02–3.77)	0.04	1.32 (0.87–2.01)	0.19
Codon 10 C (1982073)	1.58 (0.88–2.81)	0.12	1.76 (0.95–3.27)	0.07	1.37 (0.88–2.12)	0.16
Female patients (N=374)						
-509 T (1800469)	2.25 (1.06–4.76)	0.04	1.89 (0.97–3.66)	0.06	1.31 (0.85–2.00)	0.22
Codon 10 C (1982073)	3.16 (1.70–5.84)	0.0003	2.60 (1.38–4.87)	0.003	1.42 (0.91–2.20)	0.12

\* For each listed genetic variant, the higher-risk allele is indicated, and odds ratios are presented for the highest-risk genotype as compared with the lowest-risk genotype. The number of patients (804) reflects the genotypes available. Specific genotype comparisons are listed below. CI denotes confidence interval.

† The value is for the comparison of the T/T genotype with the combined C/T and C/C genotypes.

‡ The value is for the comparison of the T/T genotype with the C/C genotype.

§ The value is for the comparison of the combined C/T and T/T genotypes with the C/C genotype.

¶ The value is for the comparison of the C/C genotype with the combined C/T and T/T genotypes.

|| The value is for the comparison of the C/C genotype with the T/T genotype.

\*\* The value is for the comparison of the combined C/C and C/T genotypes with the T/T genotype.

disequilibrium with -509 and codon 10 (Fig. 1 of the Supplementary Appendix). The results indicate that these four SNPs belong to a haplotype “block” and are congruent with lower-resolution data from the International HapMap Consortium.<sup>33</sup> The Hardy–Weinberg equilibrium tests within severity groups provided further evidence of the recessive action of the codon 10 CC genotype (Results section of the Supplementary Appendix). These data are consistent with an association between disease severity and one or more founding polymorphisms in the 5' end of *TGFβ1* or immediately upstream.

**REPLICATION STUDY**

Among patients in the replication study, the distribution of sex, age, and FEV<sub>1</sub> were representative of a mixed pediatric and adult population of patients with cystic fibrosis (Table 4). Primary genetic analysis showed a strong association of the *TGFβ1* codon 10 CC genotype with lower FEV<sub>1</sub> values when patients were divided into two groups according to the FEV<sub>1</sub> value with the use of criteria derived from the initial study (FEV<sub>1</sub> at age 20, <68 percent of the predicted value or ≥68 percent) (Table 4 and Supplementary Appendix). The prevalence of the CC genotype was two times as great among patients with

an FEV<sub>1</sub> of less than 68 percent as among those with an FEV<sub>1</sub> of 68 percent or more (P=0.0002). The result was not highly sensitive to the dichotomization threshold, since P values of less than 0.01 were achieved for FEV<sub>1</sub> thresholds ranging from 65 to 74 percent of the predicted value. The secondary analysis also showed an association between codon 10 genotypes and FEV<sub>1</sub> (P=0.02); specifically, the FEV<sub>1</sub> was lower in patients with the CC genotype than in those with the TC/TT genotypes (62.8 percent vs. 68.2 percent of the predicted value). Similar results were seen among patients who were stratified according to whether they were homozygous for the ΔF508 mutation (Table 4).

**DISCUSSION**

Studies have shown that multiple genetic polymorphisms act as modifiers of lung disease in cystic fibrosis, but these studies involved small numbers of patients, patients with a broad range of *CFTR* genotypes, or limited clinical phenotyping that did not address long-term outcome (survival).<sup>4-17,19,20</sup> To increase the likelihood of identifying genetic modifiers that are relevant to the clinical outcome in cystic fibrosis, we performed two sequential studies in



**Table 4. Characteristics of Patients and *TGFβ1* Codon 10 Genotypes Stratified According to FEV<sub>1</sub> and Mean FEV<sub>1</sub> According to Codon 10 Genotype (Replication Study).\***

Variable	All Patients		ΔF508 Homozygotes	
	<68%	≥68%	<68%	≥68%
<b>CC genotype according to FEV<sub>1</sub></b>				
No. of patients	246	252	184	169
Age (yr)	20.8±8.1	22.3±7.8	21.2±8.1	22.5±7.9
Male sex (%)	48.0	57.1	48.4	53.2
Codon 10 CC genotype (%)	23.6†	11.1	21.7	10.7
	CC	TC/TT	CC	TC/TT
<b>FEV<sub>1</sub> by codon 10 genotype</b>				
No. of patients	86	412	58	295
Mean FEV <sub>1</sub> (% of predicted value)	62.8±21.3‡	68.2±22.3	62.1±21.1	67.1±23.4

\* Plus–minus values are means ±SD. The values for forced expiratory volume in one second (FEV<sub>1</sub>) are the empirical Bayes predicted values obtained for each patient at 20 years of age with the use of a mixed model, fitted to data from all 498 patients.

† P=0.0002 for the comparison with the prevalence of the CC genotype in patients with an FEV<sub>1</sub> of 68 percent or more of the predicted value, by Fisher's exact test.

‡ P=0.02 for the comparison with the mean FEV<sub>1</sub> in patients with the TC/TT genotype, by the Wilcoxon rank-sum test.

different samples of patients. The initial study tested 10 candidate modifier genes, and the replication study was performed to confirm *TGFβ1* as an important modifier in cystic fibrosis.

The initial study used an extreme phenotype design (essentially case–control) with five key features. First, to reduce genetic heterogeneity, we studied only ΔF508 homozygotes. Second, on the basis of the hypothesis that adverse (and beneficial) genetic variants would segregate with severe (and mild) lung disease, we studied patients at the extremes of lung function (lowest 25th percentile and the highest 25th percentile for age). Third, the severity of lung function was determined with the use of FEV<sub>1</sub>, the functional measure that best correlates with clinical status and outcome.<sup>34,35</sup> Fourth, the classification of pulmonary status (severe vs. mild) was confirmed by estimating final FEV<sub>1</sub> for each patient on the basis of previous measurements of FEV<sub>1</sub> during the five years before enrollment. Finally, a large number of patients were enrolled in order to improve statistical power. The sample was genetically homogeneous for ΔF508, and 96.7 percent of the patients were white; thus, population stratification is unlikely to have caused spurious association with the phenotype.<sup>36</sup>

The sample population and design of the replication study differed from the initial study in the following ways: most of the patients were from three sites (the University of North Carolina at Chapel Hill, Case Western Reserve University, or the Hospital for Sick Children, Toronto), there was

a broad range of spirometric values (i.e., patients were not selected solely from the extremes of phenotype), and the *CFTR* genotypes were mixed “pancreatic insufficient” mutations (approximately 70 percent were homozygous for the ΔF508 mutation). The primary analysis for the replication study was performed on the basis of the initial study, in which the greatest association of the CC genotype with phenotype was seen when a comparison was made between patients whose FEV<sub>1</sub> values were below 68 percent of the predicted value and those whose values were 68 percent of the predicted value or more. Despite the differences in study design, sample population, and analytic approach, the replication study clearly confirmed that the *TGFβ1* codon 10 CC genotype acted as an adverse modifier of lung disease in cystic fibrosis. Moreover, the magnitude of the effect (odds ratio, approximately 2.2) was similar to that in the initial study. The adverse *TGFβ1* genotypes that we report differ from those found in two previous smaller studies in cystic fibrosis.<sup>9,20</sup>

In addition to the biologic plausibility of *TGFβ1* as a modifier of lung disease (see below), recent association studies have linked *TGFβ1* polymorphisms to asthma and chronic obstructive pulmonary disease.<sup>29–32</sup> However, these studies involved relatively few polymorphisms, leaving open the possibility that the associations may be due to nearby causative genes in linkage disequilibrium with *TGFβ1*. Our inclusion of 31 SNPs flanking *TGFβ1* greatly narrows the possibility of a modifier gene in the region (Fig. 1). The association evidence includes the

5' end of *TGFβ1* and only one other gene (*MGC4093*) of unknown structure and function, which lies between *TGFβ1* (–509) and SNP number 7045. Transcripts of *MGC4093* have been reported in UniGene libraries of lung tissue, but Celedon et al.<sup>32</sup> argued that *MGC4093* could be ruled out as a modifier of chronic obstructive pulmonary disease on the basis of associations observed between *TGFβ1* and the disease in two samples of white patients. One sample showed the greatest evidence among 3' *TGFβ1* SNPs, and the other sample showed the greatest evidence among the 5' SNPs, –509 and codon 10. The 3' SNPs exhibit little linkage disequilibrium with the 5' SNPs,<sup>33</sup> and Celedon et al. concluded that their data implicated *TGFβ1* alone. Our genetic associations and patterns of linkage disequilibrium are consistent with the report of Celedon et al., and we propose *TGFβ1* as a likely modifier in another disease of the lung (i.e., cystic fibrosis).

The gene most likely to modify the clinical phenotype in our study, *TGFβ1*, has multiple functions related to growth and differentiation, immune responses, proinflammatory and antiinflammatory effects, and extracellular matrix production.<sup>37,38</sup> *TGFβ1* has been implicated in the pathogenesis of lung disease in animal models and humans, including disease progression in idiopathic pulmonary fibrosis, and in association with chronic obstructive pulmonary disease and asthma.<sup>29-32,39-41</sup> The association of the *TGFβ1* –509 T allele with asthma is postulated to involve increased *TGFβ1* activity, reflecting increased Yin Yang 1 binding and promoter function and accompanied by higher circulating levels of *TGF-β1*.<sup>30,31,42</sup> The *TGFβ1* polymorphisms in our study that are associated with the phenotype for severe impairment of lung function in cystic fibrosis are compatible with the hypothesis in asthma. Specifically, the –509 TT and the codon 10 CC genotypes correlate with an increase in gene expression, *TGF-β1* secretion, and circulating levels of *TGF-β1*.<sup>43-45</sup> The specific cellular- and organ-level pathophysiological mechanisms of increased *TGF-β1* activity in cystic fibrosis remain to be defined. However, this association will guide mechanistic studies and future strategies for therapeutic intervention.

In striking contrast, these same *TGFβ1* alleles (–509 T and codon 10 C) are protective against chronic obstructive pulmonary disease induced by smoking.<sup>29,32</sup> The magnitude of the increased prevalence of *TGFβ1* genotypes as adverse modifiers in our studies of cystic fibrosis and in studies of asthma,

as protective modifiers in chronic obstructive pulmonary disease,<sup>29,32</sup> is consistent—in other words, 12 to 14 percent prevalence for the –509 TT genotype and 19 to 22 percent for the codon 10 CC genotype. The contrast of *TGFβ1* polymorphisms as adverse modifiers in our studies of cystic fibrosis and in asthma, as compared with the protective effect seen in smokers, indicates that the same genetic polymorphisms may be protective or adverse, depending on environmental and other genetic factors.

In summary, we used two study designs and enrolled a large number of patients to test genes previously implicated as modifiers in cystic fibrosis. Of these candidates, only *TGFβ1* variants were strongly associated with pulmonary phenotypes that are predictive of the long-term outcome. These *TGFβ1* polymorphisms are common in cystic fibrosis, and the odds ratios for an association of the higher-risk alleles with severe disease are relatively high (>2.0) for a contribution of genetic modifiers to a mendelian disorder. Thus, genetic variation in *TGFβ1* or the immediate upstream region is an important genetic mechanism that modifies disease severity and clinical outcome in cystic fibrosis.

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## APPENDIX

In addition to the principal investigators, the following persons participated in this study: *Children's Hospital, Denver* — F. Accurso and J. Koenig; *Children's Hospital Medical Center, Cincinnati* — J.D. Acton, K. Lyons, and A. Terry; *University of Iowa* — R. Ahrens and M. Teresi; *State University of New York Upstate Medical University Hospital* — R. Anbar and D. Lindner; *Women and Children's Hospital of Buffalo* — D. Borowitz and N. Caci; *University of Alabama at Birmingham* — J.P. Clancy and V. Eubanks-Tarn; *University of Nebraska Medical Center* — J. Colombo and D. Acquazzino; *Washington University School of Medicine* — T.W. Ferkol and M. Boyle; *Drexel University College of Medicine and Morristown Memorial Hospital* — S. Fiel and P. Lomas; *Medical University of South Carolina* — P. Flume and S. Gray; *University of Virginia Medical Center* — D.K. Froh and L. Ahrens; *Nemours Children's Clinic, Orlando, Fla.* — D. Geller, K. Simpson, and K. Rinker; *Schneider Children's Hospital, New Hyde Park, N.Y.* — J. De Celie-Germana and L. Bonitz; *Medical College of Georgia* — M.F. Guill; *Texas Children's Hospital, Baylor College of Medicine* — P. Hiatt, C. Hallmark, and S. Cumming; *Michigan State University* — R. Honicky and K.L. King; *Riley Hospital for Children, Indiana University Medical Center* — M. Howenstine, M. Blagburn, and D. Terrill; *University of Cincinnati* — P. Joseph, B. Trapnell, and M. Meyers; *University of Missouri-Columbia* — P. Konig and M. Poehlmann; *Case Western Reserve University* — M. Konstan and C. Bucur; *University of North Carolina at Chapel Hill* — M.W. Leigh, K. Hohneker, and J. Robinson; *Children's Hospital, Boston* — H. Levy and I. Shempp; *University of Utah* — T. Liou, B. Marshall, and J. Jensen; *Children's Memorial Hospital, Chicago* — S.A. McColley and C. Powers; *Columbus Children's Hospital* — K. McCoy and B.M. Butera; *Mountain State Cystic Fibrosis Center* — K. Moffett and L.S. Baer; *University of Arizona* — W. Morgan and J. Douthit; *Children's Hospital and Regional Medical Center, University of Washington Medical Center, Seattle* — S. Moskowitz, R. Gibson, M. Aitken, S. McNamara, and M. Andrina; *Stanford University Medical Center* — R. Moss, C. Dunn, and Z. Davies; *University of California, San Francisco* — D.W. Nielson and D. Lallas; *Saint Louis University* — B.E. Noyes, R. Wilcott, and V. Kociela; *Wilford Hall USAF Medical Center, San Antonio* — K.N. Olivier and M. DeRosa; *Akron Children's Hospital* — G. Omlor and A. Kukay; *Antonio J. and Janet Palumbo Cystic Fibrosis Center, Children's Hospital of Pittsburgh* — D. Orenstein, S. Hurban, and E. Hartigan; *Dartmouth-Hitchcock Cystic Fibrosis Program* — H.W. Parker and B. Peterson; *University of New Mexico* — E. Perkert, H. Raissy, and C. Frantz; *Children's Hospital, Los Angeles* — A. Platzker and L. Fukushima; *Phoenix Children's Hospital* — P. Radford, A. Szpizsar Gong, and N. Argel; *University of Minnesota* — W. Regelman and J. Phillips; *University of Rochester* — C. Ren, R. Sierzega, and A.M. Kozlowski; *PriVia, The Research Centers of Via-Christi* — M. Riva, D. Dornboos, and J. Messamore; *University of Wisconsin* — M.D. Rock and L. Makhholm; *University of Mississippi Medical Center* — F. Ruiz and K. Adock; *Children's Hospital of Philadelphia* — T. Scanlin, K. Ingraham, and J. Massey; *Wake Forest University Baptist Medical Center and Rhode Island Hospital* — M.S. Schechter, M. Hunt, S. Atunah-Jay, and E. Brown; *University of Tennessee* — R. Schoumacher, P. LeNoue, and T. Rogers; *Drexel University College of Medicine* — W. Sexauer and J. Hillman; *Northern California Kaiser Permanente Medical Care Program* — G.F. Shay, G. Farmer, and M. Seastrand; *University of Michigan Health System* — R. Simon and M.E. Ball; *Emory University* — A. Stecenko and C. Cutchins; *Duke University* — J. Taylor; *Pennsylvania State University College of Medicine* — N.J. Thomas and J. Hess; *Children's Hospital of Michigan and Harper University Hospital* — D. Toder, C. Van Wagnen, and Y. LaFlore; *Toledo Children's Hospital and Toledo Hospital Cystic Fibrosis Centers* — P. Vauthy and M. Vauthy; *Saint Vincent's Hospital, Manhattan* — P. Walker, M. Berdella, and E. Langfelder-Schwind; *Monmouth Medical Center* — R.L. Zanni and B. Marra; and *Johns Hopkins University School of Medicine* — P.L. Zeitlin and L. Brass.

The following investigators and coordinators enrolled patients from Canada for the replication study: *Children's Hospital of Eastern Ontario, Ottawa* — M. Boland, T. Kovesi, and A. Smith; *Hamilton Health Science Centre, Hamilton, Ont.* — A. Freitag, L. Pedder, and R. Hennessey; *IWK Health Centre, Halifax, N.S.* — D. Hughes and P. Barrett; *Sudbury Regional Hospital, Sudbury, Ont.* — V.J. Kumar and C. Piche; *St. Paul's Adult Cystic Fibrosis Clinic, Vancouver, B.C.* — E.M. Nakielna and J. Hopkins; *Hospital for Sick Children, Toronto* — M. Solomon and L. Taylor; and *St. Michael's Hospital, Toronto* — E. Tullis and A. Tsang.

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