Genetic Modifiers of Liver Disease in Cystic Fibrosis

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Context A subset (≈3%-5%) of patients with cystic fibrosis (CF) develops severe liver disease with portal hypertension.

Objective To assess whether any of 9 polymorphisms in 5 candidate genes (α_1-antitrypsin or α_1-antiprotease [*SERPINA1*], angiotensin-converting enzyme [*ACE*], glutathione S-transferase [*GSTP1*], mannose-binding lectin 2 [*MBL2*], and transforming growth factor β1 [*TGFB1*]) are associated with severe liver disease in patients with CF.

Design, Setting, and Participants Two-stage case-control study enrolling patients with CF and severe liver disease with portal hypertension (CFLD) from 63 CF centers in the United States as well as 32 in Canada and 18 outside of North America, with the University of North Carolina at Chapel Hill as the coordinating site. In the initial study, 124 patients with CFLD (enrolled January 1999-December 2004) and 843 control patients without CFLD were studied by genotyping 9 polymorphisms in 5 genes previously studied as modifiers of liver disease in CF. In the second stage, the *SERPINA1* Z allele and *TGFB1* codon 10 genotype were tested in an additional 136 patients with CFLD (enrolled January 2005-February 2007) and 1088 with no CFLD.

Main Outcome Measures Differences in distribution of genotypes in patients with CFLD vs patients without CFLD.

Results The initial study showed CFLD to be associated with the *SERPINA1* Z allele (odds ratio [OR], 4.72; 95% confidence interval [CI], 2.31-9.61; $P=3.3\times10^{-6}$) and with *TGFB1* codon 10 CC genotype (OR, 1.53; 95% CI, 1.16-2.03; $P=2.8\times10^{-3}$). In the replication study, CFLD was associated with the *SERPINA1* Z allele (OR, 3.42; 95% CI, 1.54-7.59; $P=1.4\times10^{-3}$) but not with *TGFB1* codon 10. A combined analysis of the initial and replication studies by logistic regression showed CFLD to be associated with *SERPINA1* Z allele (OR, 5.04; 95% CI, 2.88-8.83; $P=1.5\times10^{-8}$).

Conclusions The *SERPINA1* Z allele is a risk factor for liver disease in CF. Patients who carry the Z allele are at greater risk (OR, ≈5) of developing severe liver disease with portal hypertension.

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Cystic fibrosis (CF) is a recessive monogenic disorder characterized by multiorgan involvement and clinical heterogeneity that is incompletely explained by mutations within the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene (OMIM 602421).[1] Patients with CF, including those homozygous for DF508, exhibit a range of lung disease severity, and genetic variability in non-*CFTR* genes contributes to risk for severity of pulmonary disease.[2-7]

See also Patient Page.

Intrinsic abnormalities in the liver of persons with CF reflect loss of CFTR (Cl^- channel) function on the apical membrane of cholangiocytes.[8,9] This dysfunction is predicted to result in defective (sluggish) bile flow and is associated with a cholangiocyte-induced inflammatory response with activation and proliferation of hepatic stellate cells, which results in cholangitis and fibrosis in focal portal tracts.[10-13] However, only a small fraction (≈3%-5%) of patients with CF develops severe liver disease characterized by cirrhosis with portal hypertension (CFLD)[1]; thus, non-*CFTR* genetic variability may contribute to risk for severe liver disease.[14-17]

To determine the association between non-*CFTR* genetic polymorphisms and CFLD, we studied 9 functional variants in 5 genes previously

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studied in CF liver disease, including α_1-antitrypsin (also known as α_1-antiprotease [*SERPINA1*, OMIM 107400]),[18] angiotensin-converting enzyme (*ACE* [OMIM 106180]),[19] glutathione S-transferase (*GSTP1* [OMIM 134660]),[20] mannose-binding lectin 2 (*MBL2* [OMIM 154545]),[21] and transforming growth factor β1 (*TGFB1* [OMIM 190180]).[19] Our initial study compared polymorphic genotypes in these candidate modifier genes in persons with CFLD and in control patients without CFLD aged at least 15 years. We tested our initial findings in a second study in different populations of patients with and without CFLD.

METHODS

Patients

Initial Study. Of the 158 patients with CF evaluated for CFLD (enrolled January 1999-December 2004), 128 fulfilled criteria from 22 CF centers in 10 countries (Australia [8], Canada [17], Czech Republic [17], Germany [3], Italy [28], the Netherlands [1], Scotland [2], Slovakia [4], Turkey [4], and United States [44]). For patients without 2 defined mutations in *CFTR*, we performed further testing using a panel of 70 mutations (CFTR mutation detection assay; Tm Bioscience/Luminex, Austin, Texas). After genotyping was complete, more than 95% of patients with CFLD and with 2 defined mutations in *CFTR* had 2 pancreatic insufficient mutations. The 843 control patients without CFLD were enrolled from the United States (759 from 42 centers) and Canada (84 from 32 centers). The majority of the control patients were ascertained from the GMS Lung Study population (DF508 homozygotes; 92.6%).[5] Most of the other controls had biallelic pancreatic insufficient mutations. These controls without CFLD were 15 years or older (1 SD above the mean age of diagnosis of CFLD), to exclude younger patients who might have occult liver disease.

Replication Study. Of the 191 patients with CF evaluated for CFLD (enrolled January 2005-February 2007), 139 fulfilled criteria from 35 CF centers in 10 countries (Argentina [5], Australia [5], Canada [24], Chile [1], England [4], France [9], Ireland [8], Israel [7], Italy [14], and United States [62]). The percentage of pancreatic insufficient *CFTR* genotypes in patients with CFLD was similar to those in the initial study. The 1088 control patients (≥15 years and without CFLD) were ascertained from 5 countries (Canada [391 from 32 centers], Czech Republic [30 patients], Ireland [6 patients], Italy [71 patients], and United States [590 from 54 centers]). The majority of the controls had 2 pancreatic insufficient mutations (93.5%; mostly DF508/DF508 [62.8%]).

Enrollment Criteria

All patients had a diagnosis of CF, confirmed by sweat test, *CFTR* genotyping, or both. CFLD was defined as cirrhosis in patients 2 years or older, confirmed by imaging (ultrasound, computed tomography, magnetic resonance imaging) showing hepatic parenchymal abnormalities and portal hypertension (esophageal varices, portal-systemic collaterals, splenomegaly) in the absence of another cause for liver disease. Data were independently reviewed by 2 hepatologists (S.C.L., P.R.D.) with experience in CFLD to ensure inclusion and exclusion criteria were met, using case report forms, radiology and endoscopy reports, and clinical notes. When there was no consensus, the reviewers requested additional information to clarify the diagnosis of CFLD. No patient was excluded because of race or ethnicity, defined by patient self-report.

We excluded 30 (19%) and 52 (27%) patients originally submitted for the initial and replication studies, respectively, with a presumed diagnosis of CFLD, because they had milder liver disease without portal hypertension or inadequate documentation. For the 47 patients with confirmed CFLD who had undergone a liver transplant (26 in initial study; 21 in replication study), source documents were obtained from dates prior to transplantation. Exclusion criteria for the CFLD group included portal vein thrombosis or other causes of liver disease (alcohol abuse, biliary atresia, clinically significant viral hepatitis, use of parenteral nutrition, and Wilson disease). The study was approved by the institutional review boards of all participating institutions; all participants (or their parent) provided written informed consent.

Exclusion From Analysis Based on Age at Diagnosis of CFLD

In common with previous reports, we found the mean age of diagnosis of CFLD (first documentation of portal hypertension) to be 10.6 (SD, 5.4) years.[15,22-25] The diagnosis of CFLD was first established after age 30 years in 7 patients (aged 32, 33, 35, 40, 43, 44, and 47 years), which is 4 or more SDs above the mean of the normal distribution. Therefore, these patients were excluded from the genetic analyses (4 from the initial study, 3 from the replication study).

Data Collection and Laboratory Methods

Patients received a unique identifier code, and data were stored in a secure database in the UNC Bioinformatics Center. Clinical data on standard case report forms included self-reported race/ethnicity, pancreatic exocrine status, medical history, physical examination, laboratory blood work values, and abdominal radiology reports. In addition, we reviewed the following procedure reports if available: liver explant pathology (from liver transplantation), liver biopsy, endoscopy, and colonoscopy.

DNA was extracted from peripheral blood leukocytes using standard protocols.[26] Genetic polymorphisms were determined by direct sequencing, microsphere-based genotyping using Illumina BeadArray technology (Illumina Inc, San Diego, California), and site-directed mutagenesis.

Immunohistochemistry with polyclonal rabbit anti–α_1-antitrypsin antibody and monoclonal mouse anti-CD68 (clone KP1) antibody (Dako Canada Inc, Mississauga, Ontario, Canada), was performed on the Benchmark XT autoimmunostainer (Ventana Medical Systems, Tucson, Arizona) at dilutions of 1:3000 and 1:5000, respectively. Immunodetection was per-

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formed using the Ventana i-VIEW DAB, LSAB kit. Tissue sections were dewaxed, enzyme pretreated for α_1-antitrypsin, heat epitope retrieved for CD68, peroxidase, and endogenous biotin blocked using Ventana proprietary reagents. Sections were hematoxylin-eosin counterstained for nuclear detail.

Statistical Analysis

Genotype distributions were tested for consistency with expected Hardy-Weinberg equilibrium proportions for case and control patients in the initial, replication, and combined studies, using all patients and then restricted to white patients, using PLINK version 1.03 (http://pngu.mgh.harvard.edu/~purcell/plink/).[27]

For the initial study, the association between polymorphisms and CFLD was assessed using Cochran-Armitage trend tests.[28] All tests were 2-sided; unadjusted *P* values are reported, along with *P* values that were significant ($P < .05$) after Bonferroni correction adjusting for 9 tests. Analyses were performed using all patients and then restricted to white patients.

For the replication study, the association between 2 polymorphisms from the initial study (*SERPINA1* Z allele and *TGFB1* codon 10) and CFLD was assessed using Cochran-Armitage trend tests. Initial and replication samples were subsequently combined and analyzed for the *SERPINA1* Z allele using Cochran-Armitage trend tests and logistic regression models. Varying levels of covariate adjustment in the logistic regression models were made for ethnicity (as a 5-level categorical variable for all samples), sex, *CFTR* genotype, and *TGFB1* codon 10 genotype. Tests of interactions were performed to assess whether the odds of CFLD differed between male and female patients by *SERPINA1* genotype. Odds ratios (ORs), corresponding 95% confidence intervals (CIs), and uncorrected *P* values are reported. Bonferroni correction was applied to assess overall statistical significance in the replication and combined analyses (adjusting for 2 tests in the replication and 9 tests in the combined sample). Analyses were performed separately, first using all samples and then using those from white patients only.

Analysis of variance models were used to assess whether sex, *SERPINA1* Z allele, and *CFTR* genotype were associated with age of diagnosis of CFLD in the combined sample. Data were analyzed on all CFLD case patients with covariate adjustment for self-reported ancestry, and on white CFLD case patients with a reported age at diagnosis.

To estimate population attributable risk, we used a modified form of the classic Levin formula for population attributable fraction by replacing relative risk estimates with ORs and using the proportion of control patients carrying the Z allele as an estimate of the probability of exposure.[29-31]

While this estimate is not exact, given our case-control sampling design (oversampled older patients without CFLD), this estimate should provide a reasonable approximation, owing to the modest frequency of CFLD in patients with CF ($\approx$5%).

RESULTS

Clinical Features

Initial Study. Characteristics of the initial group of 124 patients with CFLD and 843 patients without CFLD are shown in TABLE 1. The CFLD group was younger at enrollment, had more male patients, and had slightly fewer white patients. The *CFTR* mutations in patients with CFLD were representative of pancreatic insufficient mutations in North American and European patients with CF (Table 1).[1] The prevalence of meconium ileus at birth in patients with CFLD (18.2%) is comparable to that in the control group and typical for the general CF population with pancreatic insufficient *CFTR* mutations.[1]

Abnormalities in biochemical tests of the liver (aspartate transaminase, alanine transaminase, and γ-glutamyl transferase) were not predictive of CFLD and also were not markers of hepatocellular synthetic dysfunction, such as international normalized ratio and levels of serum albumin (TABLE 2). Preoperative assessment of data available from a subset of patients (n=22) who underwent liver transplantation (n=43) showed a distribution of abnormal total bilirubin and albumin values similar to that of the nontransplanted patients (Table 2).

Replication Study. Based on the associations for the *SERPINA1* Z allele and *TGFB1* codon 10 (TABLE 3), we enrolled additional patients with and without CFLD to test for replication (TABLE 4). The characteristics of the replication patients were similar to those in the initial study (Table 1), including the distribution of specific *CFTR* mutations, prevalence of meconium ileus (23.8%), and liver function abnormalities (Table 2 and Table 4).

Cochran-Armitage Trend Test of Association

Initial Study. In the analysis of previously studied gene modifiers of liver disease in CF (Table 3), association was seen only for the *SERPINA1* Z allele (OR, 4.72; 95% CI, 2.31-9.61; $P = 3.3 \times 10^{-6}$) and *TGFB1* codon 10 (OR, 1.53; 95% CI, 1.16-2.03; $P = 2.8 \times 10^{-3}$). The *SERPINA1* Z allele displayed association for all patients with CFLD but was more prominent in female patients. Similar results were seen when the analysis was restricted to white patients (data not shown). It is noteworthy that small effects for the nonsignificant polymorphisms would not be detected with sufficient power by this study. The genotypes and minor allele frequencies for genetic variants in patients without CFLD were similar to those previously reported.[5-7,18-21]

Replication Study. The association was replicated for the *SERPINA1* Z allele (OR, 3.42; 95% CI, 1.54-7.59; $P = 1.4 \times 10^{-3}$) (TABLE 5), but the association was more prominent in male patients, in contrast to the initial study. Similar results were seen when analyses were restricted to white patients (data not shown). The association of the *TGFB1* codon 10 variant was not replicated for all patients (Table 5) or for male or female patients when analyzed separately (data not shown).

Initial Plus Replication Study. When the initial and replication populations were combined for analysis using

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Cochran-Armitage trend tests, the *SERPINA1* Z allele displayed very robust association with CFLD (OR, 4.17; 95% CI, 2.46-7.05; $P=9.9\times10^{-9}$); similar evidence for association was observed in analyses restricted to white patients in the initial plus replication populations (data not shown).

Hardy-Weinberg Equilibrium

All polymorphisms had genotype distributions consistent with Hardy-Weinberg equilibrium ($P>.01$) in the initial, replication, and combined samples, irrespective of how samples were partitioned according to ethnicity and CFLD status.

Logistic Regression for the Z Allele

We combined the initial and replication groups and performed logistic regression for the *SERPINA1* Z allele to estimate the odds of CFLD, adjusting for the covariates of ethnicity, sex, and *CFTR* genotype. Results remained consistent when using all patients or white patients only, with respect to statistical significance estimates ($P=1.5\times10^{-8}$ or $P=6.3\times10^{-8}$, respectively) as well as OR estimates (OR, 5.04; 95% CI, 2.88-8.83 for all patients vs OR, 4.87; 95% CI, 2.75-8.64 for white patients only). In addition, we saw no evidence for interactions between sex and the *SERPINA1* Z allele in all patients or only white patients. Similar results were obtained by logistic regression adjusting only for ethnicity in the complete sample and in models that additionally adjusted for the *TGFB1* codon 10 genotype.

Population Attributable Risk

We combined the initial and replication groups and estimated the population attributable risk for the Z allele to be 6.7% (white patients only, 6.6%). A similar result was obtained using another method of estimating the probability of exposure, namely the average Z allele frequency of patients from North America, Europe, and Australia (data not shown).

Age at Diagnosis of CFLD

The mean and median age of recognition (diagnosis) of portal hypertension in all patients with CFLD was approxi-

Table 1. Initial Study: Characteristics of Patients With Cystic Fibrosis With or Without Severe Liver Disease

Variable	Liver Disease (n = 124)	No Liver Disease (n = 843)
Age at enrollment, mean (SD), [median], y	19.8 (7.3) [18.8]	26.7 (9.6) [23.3]
Male sex, No. (%)	88 (71.0)	462 (54.8)
White race, No. (%)	115 (92.7)	822 (97.5)
Genotype, No. (%)[a]		
PI/PI	100 (80.7)	836 (99.2)
PI/PS	5 (4.0)	0
PS/PS	0	0
PI/unknown	16 (12.9)	7 (0.8)
Unknown/unknown	3 (2.4)	0
Meconium ileus, No. (%)[b]	22 (18.2)	68 (16.5)
Age of diagnosis of portal hypertension, y[c]		
Mean (SD)	10.3 (5.9)	NA
Median (range)	10 (0.5-26)	NA
Portal hypertension documented by, No. (%)[d]		
Splenomegaly	120 (97.1)	NA
Varices (esophageal, rectal)	93 (74.6)	NA
Hypersplenism[e]	69 (62.7)	NA

Abbreviations: NA, not applicable; PI, pancreatic exocrine insufficient mutation; PS, pancreatic exocrine sufficient mutation.
[a] *CFTR* mutations for patients with cystic fibrosis and liver disease in initial study: DF508/DF508 (56.5%), DF508/PI (19.4%), DF508/unknown (10.5%), PI/PI (4.8%), PI/unknown (2.4%), PI/PS (4.0%), unknown/unknown (2.4%). *CFTR* mutations for patients with cystic fibrosis and no liver disease in initial study: DF508/DF508 (92.6%), DF508/PI (5.9%), DF508/unknown (0.7%), PI/PI (0.7%), PI/unknown (0.1%).
[b] Data available from 121 patients with cystic fibrosis and liver disease (aged 0-26 years) and 411 patients with cystic fibrosis and no liver disease (aged 15-28 years).
[c] Data available from 122 patients with cystic fibrosis and liver disease.
[d] Some patients had portal hypertension confirmed by more than 1 method; all patients tested had findings compatible with multilobular cirrhosis.
[e] As defined by platelet count less than 100 000 cells/μL; data available for 110 patients.

Table 2. Summary of Clinical Laboratory Values for Patients With Cystic Fibrosis and Severe Liver Disease

Measure	No. of Patients[a]	% of Patients: Normal Range	>1× to ≤2× Upper Limit	>2× Upper Limit
Aspartate transaminase				
Range of values		≤30 U/L	31-60 U/L	>60 U/L
Initial	122	23.0	43.4	33.6
Replication	132	16.7	47.7	35.6
Alanine transaminase				
Range of values		≤40 U/L	41-80 U/L	>80 U/L
Initial	116	47.4	35.3	17.2
Replication	133	44.4	37.6	18.0
γ-Glutamyl transferase				
Range of values		≤30 U/L	31-60 U/L	>60 U/L
Initial	110	24.5	16.4	59.1
Replication	114	19.3	28.1	52.6
Total bilirubin[b]				
Range of values		≤1.2 mg/dL	1.3-2.4 mg/dL	>2.4 mg/dL
Initial	106	66.0	18.9	15.1
Replication	111	70.3	17.1	12.6
Albumin[c]				
Range of values		≥3.5 g/dL	Low: 2.5-3.4 g/dL	Very low: <2.5 g/dL
Initial	104	49.0	42.3	8.7
Replication	120	56.7	39.2	4.1
International normalized ratio				
Range of values		<1.2	Moderately high: 1.2-1.5	High: >1.5
Initial	88	28.4	51.1	20.5
Replication	90	32.2	47.8	20.0

[a] Number of patients with data available.
[b] Total bilirubin level abnormal in 9 of 22 patients (40.9%) in initial study and 8 of 21 patients (38.1%) in replication study, just prior to liver transplantation.
[c] Albumin level abnormal in 13 of 21 patients (61.9%) in initial study and 10 of 20 patients (50.0%) in replication study, just prior to liver transplantation.

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mately 10 to 11 years, and 90% of patients had CFLD diagnosed before age 20 years. Male patients had an earlier age of diagnosis of CFLD than female patients for all patients (males, 8.5 years; females, 10.5 years; $P = .007$), and for white patients (males, 9.7 years; females, 11.5 years; $P = .03$). Age at diagnosis of CFLD was not associated with the presence of the *SERPINA1* Z allele, *CFTR* genotype, or self-reported ancestry.

Liver Histopathology

A patient with CFLD carrying a single copy of the *SERPINA1* Z allele accumulated SERPINA1 protein within hepatocytes adjoining the fibrosed portal tracts, but SERPINA1 protein was not seen in hepatocytes of a patient with CFLD and without the Z allele.

COMMENT

Previous studies have suggested that genetic polymorphisms may act as modifiers of liver disease in cystic fibrosis, but these studies were small and phenotyping did not address the development of severe (biliary) cirrhosis associated with portal hypertension.[18-21] To increase the likelihood of identifying genetic modifiers relevant to the development of severe liver disease in CF, ie, cirrhosis with portal hypertension, we performed 2 sequential studies in different groups of patients. The initial study involved 5 candidate genes that had previously been studied as modifiers of CF liver disease,[18-21] and the replication study tested for confirmation of *SERPINA1* Z allele and *TGFB1* codon 10 variant as modifiers of severe liver disease in CF.

This study had 3 key design features. First, we used rigorous criteria to identify patients with CF and portal hypertension (cases), reflecting hepatobiliary cirrhosis, and key source documents were reviewed independently by 2 experts to confirm the CFLD phenotype. Second, for patients without CFLD (controls), we studied only those 15 years or older, to exclude younger patients with predisposition to develop CFLD. Third, we enrolled a large number of patients with and without CFLD to improve statistical power. For the initial study, approximately 50% of the case patients were from outside North America, and 93% were self-described as white; all control patients were from North America. For the replication (second) study, a slightly greater percentage of case patients were from North America (63% vs 50%), and some control patients (10%) were from outside North America.

Genetic analyses of the initial cohort showed that a single copy of the *SERPINA1* Z allele and each additional copy of the *TGFB1* codon 10 C allele were associated with significantly increased odds of CFLD. In the replication study, the *SERPINA1* Z allele was confirmed as a modifier of liver disease in CF, whereas the *TGFB1*

Table 3. Initial Study: Prevalence of Polymorphic Genotypes in Patients With Cystic Fibrosis With or Without Severe Liver Disease

Gene/Variant[a]	SNP No.	Liver Disease	Genotype	No. (%) With Genotype	Genotype	No. (%) With Genotype	Genotype	No. (%) With Genotype	No. Genotyped	*P* Value[b]	OR (95% CI)[c]
SERPINA1											
S allele (T2313A)	rs17580	Yes	AA	90 (88.2)	AT[d]	12 (11.8)	TT[e]	0	102	.16	1.59 (0.83-3.05)
		No	AA	619 (92.6)	AT[d]	49 (7.3)	TT[e]	1 (0.1)	669		
Z allele (G4627A)	rs28929474	Yes	GG	110 (88.7)	AG[f]	14 (11.3)	AA[g]	0	124	3.3×10^{-6}[h]	4.72 (2.31-9.61)
		No	GG	741 (97.4)	AG[f]	20 (2.6)	AA[g]	0	761		
ACE D/I deletion (T2313A)	NA	Yes	DD	43 (35.0)	DI	54 (43.9)	II	26 (21.1)	123	.45	1.11 (0.85-1.44)
		No	DD	250 (37.3)	DI	300 (44.7)	II	121 (18.0)	671		
GSTP1 (A1375G)	rs1695	Yes	AA	40 (41.7)	AG	41 (42.7)	GG	15 (15.6)	96	.32	1.17 (0.86-1.61)
		No	AA	316 (43.7)	AG	331 (45.8)	GG	76 (10.5)	723		
MBL2											
O	NA	Yes	AA	69 (59.0)	AO	42 (35.9)	OO	6 (5.1)	117	.92	0.98 (0.70-1.38)
		No	AA	384 (57.9)	AO	248 (37.4)	OO	31 (4.7)	663		
XA/O	NA	Yes	Other	95 (82.6)	XA/O	14 (12.2)	O/O	6 (5.2)	115	.50	1.14 (0.78-1.65)
		No	Other	567 (85.5)	XA/O	65 (9.8)	O/O	31 (4.7)	663		
TGFB1											
Promoter (C-509T)	rs1800469	Yes	CC	44 (39.6)	CT	52 (46.9)	TT	15 (13.5)	111	.01	1.45 (1.07-1.95)
		No	CC	413 (49.6)	CT	356 (42.7)	TT	64 (7.7)	833		
Codon 10 (C29T)	rs1800470	Yes	TT	33 (29.5)	CT	54 (48.2)	CC	25 (22.3)	112	2.8×10^{-3}[i]	1.53 (1.16-2.03)
		No	TT	343 (40.7)	CT	390 (46.4)	CC	109 (12.9)	842		
Codon 25 (G74C)	rs1800471	Yes	GG	93 (83.8)	GC	18 (16.2)	CC	0	111	.71	1.10 (0.66-1.85)
		No	GG	592 (85.9)	GC	92 (13.4)	CC	5 (0.7)	689		

Abbreviations: CI, confidence interval; NA, not applicable; OR, odds ratio; SNP, single-nucleotide polymorphism.
[a] See "Methods" for gene accession numbers.
[b] Calculated using Cochran-Armitage trend test of comparisons of the genotypes.
[c] For each additional copy of the minor allele.
[d] Heterozygous form of the S allele.
[e] Homozygous form of the S allele.
[f] Heterozygous form of the Z allele.
[g] Homozygous form of the Z allele.
[h] Bonferroni-corrected $P = 3.0 \times 10^{-5}$.
[i] Bonferroni-corrected $P = .03$.

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codon 10 variant was not confirmed. It is noteworthy that small effects of the nonsignificant polymorphisms in other genes would not be detected with sufficient power by this study. The association of the *SERPINA1* Z allele with CFLD contrasts with results from a previous "negative" study that used less stringent phenotypic markers of CF liver disease, such as liver function tests, which do not correlate with severity of CFLD (portal hypertension).[18]

When the initial and replication study populations were combined for joint analysis by multivariable logistic regression, the magnitude of the effect of the *SERPINA1* Z allele was large compared with most genetic association studies (OR, ≈5) when sex, ethnicity, and *CFTR* genotype were included as covariates. The strength of the association of the *SERPINA1* Z allele with CFLD varied by sex for the initial vs the replication studies, but the overall odds were not statistically different for female and male patients when all patients were analyzed. Population stratification is unlikely to account for the results for the *SERPINA1* Z allele; the prevalence of the Z allele (1.14%) in patients without CFLD (controls) in our study is similar to that reported for more than 85 000 individuals genotyped in pertinent regions of the world (1.20%).[32,33]

The mechanism of the *SERPINA1* Z allele as an adverse modifier of liver disease in patients with CF likely reflects the dual stimulation of hepatic stellate cells by inflammatory mediators from *CFTR*-deficient cholangiocytes as well as hepatocytes containing the misfolded SERPINA1 protein, ie, these inflammatory stimuli induce hepatic stellate cells to migrate and proliferate in the bile duct regions in a profibrogenic manner.[10-13,16,34-38] Bile duct ligation with resultant cholestasis induces more activated stellate cells and fibrosis in the liver of homozygous transgenic PiZ vs wild-type mice, which is compatible with this proposed mechanism of the Z allele as an adverse modifier in CF.[39] Further studies are necessary to better define the pathogenesis of the Z allele in CFLD.

The Z allele variant causes misfolding of the SERPINA1 protein, which results in an accumulation of protein in hepatocytes. The most prevalent *CFTR* mutation, DF508, is also a misfolding mutation, expressed predominantly in cholangiocytes in the liver.[8-13,34-38] However, it is unlikely that folding mutations in *CFTR* and *SERPINA1* induce an amplified, adverse effect on the proteosomal degradation pathway, because these 2 genes are predominantly expressed in 2 different cell types in the liver.[8,9,34-38] Furthermore, heterozygosity for the Z allele is associated with the risk and progression of a variety of liver diseases, including cryptogenic cirrhosis, biliary atresia, viral hepatitis, alcoholic cirrhosis, and nonalcoholic fatty liver disease.[37,38,40]

By studying a large number of patients with CF and with well-defined severe liver disease and portal hypertension, we confirmed and refuted some previous observations and discovered new information about the clinical features of these patients. We confirmed that (1) CFLD is more common and is diagnosed earlier in male individuals; (2) specific *CFTR* mutations do not correlate

Table 4. Replication Study: Characteristics of Patients With Cystic Fibrosis With or Without Severe Liver Disease

Variable	No. (%) Liver Disease (n = 136)	No. (%) No Liver Disease (n = 1088)
Age at enrollment, mean (SD) [median], y	18.2 (6.2) [16.6]	27.2 (9.2) [25.0]
Male sex	82 (60.3)	566 (52.0)
White	125 (91.9)	1066 (98.0)
Genotype[a]		
PI/PI	116 (85.4)	1017 (93.5)
PI/PS	4 (2.9)	13 (1.2)
PS/PS	0	2 (0.2)
PI/unknown	14 (10.3)	44 (4.0)
Unknown/unknown	2 (1.4)	12 (1.1)
Meconium ileus[b]	31 (23.8)	62 (18.3)
Age at diagnosis of portal hypertension[c]		
Mean (SD), y	11.0 (4.7)	NA
Median (range)	11 (0.5-28)	NA
Portal hypertension documented by		
Splenomegaly[d]	124 (91.1)	NA
Varices (esophageal, rectal)	101 (74.2)	NA
Hypersplenism[e]	52 (44.4)	NA

Abbreviations: NA, not applicable; PI, pancreatic exocrine insufficient mutation; PS, pancreatic exocrine sufficient mutation.

[a] *CFTR* mutations for patients with cystic fibrosis and liver disease in replication study: DF508/DF508 (45.6%), DF508/PI (32.4%), DF508/unknown (9.6%), PI/PI (7.4%), PI/unknown (0.7%), PI/PS (2.9%), and unknown/unknown (1.4%). *CFTR* mutations for patients with cystic fibrosis and no liver disease in replication study: DF508/DF508 (62.8%), DF508/PI (27.5%), DF508/PS (0.6%), DF508/unknown (3.7%), PI/PI (3.1%), PI/unknown (0.4%), PI/PS (0.6%), PS/PS (0.2%), and unknown/unknown (1.1%).

[b] Data available from 130 patients with cystic fibrosis and liver disease (aged 0-28 years) and 339 patients with cystic fibrosis and no LD (aged 15-28 years).

[c] Data available from 120 patients with cystic fibrosis and liver disease.

[d] Some patients had portal hypertension confirmed by more than 1 method; all patients tested had findings compatible with multilobular cirrhosis.

[e] As defined by platelet count less than 100 000 cells/μL; data available for 117 patients.

Table 5. Replication Study: Prevalence of Polymorphic Genotypes in Patients With Cystic Fibrosis With or Without Severe Liver Disease

Gene/Variant	SNP No.	Liver Disease	Genotype	No. (%) With Genotype	Genotype	No. (%) With Genotype	Genotype	No. (%) With Genotype	No. Genotyped	*P* Value[a]	OR (95% CI)[b]
SERPINA1 Z allele (G4627A)	rs28929474	Yes	GG	127 (93.4)	AG[c]	9 (6.6)	AA[d]	0	136	1.4×10^{-3}[e]	3.42 (1.54-7.59)
		No	GG	1062 (98.0)	AG[c]	22 (2.0)	AA[d]	0	1084		
TGFB1 codon 10 (C29T)	rs1800470	Yes	TT	51 (38.1)	CT	62 (46.2)	CC	21 (15.7)	134	.96	1.01 (0.77-1.31)
		No	TT	290 (38.3)	CT	349 (46.1)	CC	118 (15.6)	757		

Abbreviations: CI, confidence interval; OR, odds ratio; SNP, single-nucleotide polymorphism.

[a] Calculated using Cochran-Armitage trend test of comparisons of the genotypes.

[b] For each additional copy of the minor allele.

[c] Heterozygous form of the Z allele.

[d] Homozygous form of the Z allele.

[e] Bonferroni-corrected $P = 2.8 \times 10^{-3}$.

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with CFLD, but *CFTR* mutations with residual function (pancreatic sufficient mutations) are uncommon in individuals with CFLD; (3) hepatic synthetic function is preserved for long durations in most patients with CFLD; (4) thrombocytopenia due to hypersplenism is common in individuals with portal hypertension due to CFLD; (5) liver biochemical tests are poorly predictive of severe liver disease and portal hypertension in CF; and (6) severe liver disease with portal hypertension develops in pediatric patients by age 10 to 12 years.[14-17,22-25] In addition, we made a striking observation about the age distribution of diagnosis of severe liver disease, whereby the prevalence of severe liver disease does not increase in adults with CF, despite progressive increase in longevity; more than 90% of the patients with CFLD in our study were diagnosed by age 20 years, with a mean (and median) age of diagnosis of 10 to 11 years. We were not able to confirm any association of meconium ileus with CFLD, as has been reported in some,[15,16,22-25] but not all,[17,41] studies; the prevalence of meconium ileus in patients with CFLD in our study (≈21%) was similar to that reported for patients with pancreatic exocrine insufficiency.[1]

In summary, we studied 2 large populations of patients with CF with and without liver disease and portal hypertension to test genes previously studied as modifiers of liver disease. Of these candidate genes, only the *SERPINA1* Z allele was significantly associated with CFLD and portal hypertension. This polymorphism is relatively uncommon in CF (≈2.2% of patients with CF are carriers), but the OR for association with severe liver disease is relatively high (≈5) for the contribution of a genetic modifier to a mendelian disorder. Moreover, the estimated population attributable risk among patients with CF is 6.7%. From a clinical perspective, a rare variant with large penetrance (such as the Z allele) may be more useful than a common variant with low penetrance in screening for genetic polymorphisms. The identification of the *SERPINA1* Z allele as the first marker for the development of severe liver disease in CF illustrates the possibility of identifying CF risk factors early in life, conceptually as a secondary component of neonatal screening after the diagnosis of CF is confirmed.

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