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Relative Contribution of Genetic and Non-genetic Modifiers to Intestinal Obstruction in Cystic Fibrosis

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Abstract

Background & Aims—Neonatal intestinal obstruction (meconium ileus or MI) occurs in 15% of patients with cystic fibrosis (CF). Our aim was to determine the relative contribution of genetic and non-genetic modifiers to the development of this major complication of CF.

Methods—Using clinical data and DNA collected by the CF Twin and Sibling Study, 65 monozygous twin pairs, 23 dizygous twin/triplet sets, and 349 sets of siblings with CF were analyzed for MI status, significant covariates, and genome-wide linkage.

Results—Specific mutations in *CFTR*, the gene responsible for CF, correlated with MI indicating a role for *CFTR* genotype. Monozygous twins showed substantially greater concordance for MI than dizygous twins and siblings ($p=1\times 10^{-5}$) demonstrating that modifier genes independent of *CFTR* contribute substantially to this trait. Regression analysis revealed that MI was correlated with distal intestinal obstruction syndrome (DIOS; $p=8\times 10^{-4}$). Unlike MI, concordance analysis indicated that the risk for development of DIOS in CF patients is primarily due to non-genetic factors. Regions of suggestive linkage (logarithm of the odds of linkage >2.0) for modifier genes that cause MI (chromosomes 4q35.1, 8p23.1, and 11q25) or protect from MI (chromosomes 20p11.22 and 21q22.3) were identified by genome-wide analyses. These analyses did not support the existence of a major modifier gene within the CFM1 region on chromosome 19 that had previously been linked to MI.

Conclusions—The *CFTR* gene along with two or more modifier genes are the major determinants of intestinal obstruction in newborn CF patients, while intestinal obstruction in older CF patients is primarily due to non-genetic factors.

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Keywords

Twins; siblings; linkage; association; intestinal obstruction; CFM-1

Introduction

Cystic fibrosis (CF [MIM 219700]) is an autosomal recessive disease caused by mutations in the CF transmembrane regulator (CFTR [MIM 602421]). Variation in the *CFTR* genotype has been associated with some aspects of the CF phenotype such as pancreatic status (1). On the other hand, many other variable features of CF are not correlated, indicating that factors independent of *CFTR* genotype play a major role in the complications and course of this “single gene” disorder (2;3). Two such features are meconium ileus (MI), a form of intestinal obstruction observed in the neonatal period that occurs in 13–20% of CF patients (3–6) and distal intestinal obstruction syndrome (DIOS), a trait with clinical and pathologic similarities to MI that affects older CF patients (7;8). Estimates of the prevalence of DIOS in CF patients vary considerably (2.4% to 41.3%) probably due to differences in the diagnostic criteria, length of follow-up and age range of the patients studied (7–10). Several reports indicate that the recurrence rate of MI in siblings is significantly higher than the prevalence of MI in unrelated patients suggesting that factors shared among siblings contribute to the development of MI (4;11–13). However, it is not known whether familial concordance of MI is the result of shared genetic or environmental factors or a combination of both.

Evidence for genetic factors underlying intestinal obstruction has been derived from studies of the CF mouse model. The majority of homozygous CFTR null mice have a severe and usually fatal intestinal obstruction in early life (14). By capitalizing on strain-specific differences in survival among CFTR null mice, Rozmahel and colleagues were able to locate a modifier locus to the distal portion of mouse chromosome 7 (15). Several other loci for survival from intestinal obstruction in CF mice have since been identified (16). Zielenski and coworkers postulated that the locus on chromosome 7 responsible for murine intestinal obstruction may contain a gene that is responsible for MI in CF patients. This theory was tested by genotyping 185 North American CF sibling pairs and parents using 9 microsatellite markers that spanned 7.65 Mb on chromosome 19q13, a region of conserved synteny with the segment of mouse chromosome 7 that was linked to intestinal obstruction. Several markers from this region demonstrated significant linkage with the MI phenotype consistent with the presence of a MI modifier gene (CFM1 [MIM 603855]) within this region (17). However, the gene (or genes) within the CFM1 region that modifies MI has not been identified.

The CF Twin and Sibling Study in the United States is currently recruiting patients to assess genetic and non-genetic contribution to disease variation in CF. Monozygous (MZ) and dizygous (DZ) twins can differentiate the relative contribution of genetic factors since twins have a high degree of shared environment, but MZ and DZ twins differ in their degree of gene sharing (100% versus 50%) (18–21). On the other hand, siblings have the same degree of gene sharing as DZ twins (50%), but a lower degree of shared environment so that a comparison of DZ twins and siblings can estimate environmental contribution. Although recruitment for the CF Twin and Sibling study is not complete, MI and DIOS are sufficiently frequent to perform an analysis of concordance rates among the patients recruited to date. We also performed an initial genome-wide linkage analysis for MI including a re-evaluation of the CFM1 locus.

Materials and Methods

Family collection

The CF Twin and Sibling Study is a collaborative effort to collect detailed clinical information and DNA samples from affected siblings and their parents. Informed consent was obtained from all subjects prior to enrollment in the study. Enrollment was based on conclusive diagnosis of CF (22). The diagnosis of MI was based on the presence of the following features in the newborn period: lack of passage of stool within 24 hours after birth, evidence of obstruction on abdominal X-ray (ground glass appearance of intestine, air fluid levels and/or intra-abdominal calcifications), evidence of colonic abnormality (microcolon on X-ray) and treatment for obstruction (enema or surgery). Some cases were complicated by bilious vomiting, perforation and/or atresia of jejunum or ileum. The diagnosis of DIOS was based upon the clinical impression of the reporting CF center. A required feature was intestinal obstruction requiring treatment beyond laxatives (e.g., oral polyethylene glycol solution, enema or surgery); radiological documentation of DIOS was obtained for some, but not all patients. Pancreatic status was obtained from patient medical records. Pancreatic insufficient status was determined by a fecal fat test, a fecal elastase test, the appearance of the stool pattern, the presence of oil in the stool, the patient's growth pattern, and/or the complaint of abdominal pains.

DNA isolation and genotyping methods

Genomic DNA was isolated from whole blood using the phenol/chloroform procedure (23). Zygosity status of twins was determined by AmpFISTR Profiler (Applied Biosystems, Foster City, CA). *CFTR* genotype was obtained from the patient's medical record. In cases where *CFTR* genotyping had not been performed or was incomplete, DNA samples were typed for 58 *CFTR* alleles using the Roche PCR-based Line Probe Assay (Roche Molecular Systems, Alameda, CA) (24). DNA samples with one or more unidentified *CFTR* mutations following typing with the Line Probe Assay were subjected to DNA sequencing of all coding regions of *CFTR* and flanking introns (25). For genome-wide linkage studies, patients were genotyped by the Marshfield Genotyping Center for 402 polymorphic short tandem repeat markers with an average spacing of 10 cM, or 7.5 Mb. The average rate of genotypes called for the 1161 individuals was 95% (range 70–99.8%); identified mendelian errors numbered 642 (1.1%). Average heterozygosity in the study population was 75% (range 57–89%). SNP typing was performed using 100K SNP chips from Affymetrix (26); 90,689 markers met quality criteria for inclusion (minor allele frequency at least 1%; genotype obtained in at least 85% of samples) and yielded an average marker spacing of 23.6 kilobases. The average rate of genotypes called for the 86 individuals was 96% (range 80–100%); identified mendelian errors numbered 2115 (0.03%). Average heterozygosity was 30%. For the chromosome 19-specific map, patients were genotyped at 7 STR markers spanning 6.5 Mb of chr19q13: D19S211, D19S217, D19S219, D19S112, D19S412, D19S902, and D19S604 yielding an average marker spacing of 1.1 Mb. STR markers were sized using an ABI Prism 310 DNA Sequencer or an ABI 3100 DNA Sequencer (Applied Biosystems) and GENESCAN analysis software version 3.1.2. Genotyping was internally controlled by genotyping individuals of the same family on the same run. Any ambiguous genotypes and genotypes that were not consistent with mendelian inheritance were re-typed.

Concordance Analysis

Concordance for disease (MI or DIOS) was calculated by the number of pairs concordant for disease divided by the total number of pairs in which at least one member had disease. Concordance for lack of disease was calculated by the number of pairs in which neither member had disease divided by the total number of pairs in which at least one member did not have disease. Heritability estimates were estimated as described by Falconer (27;28). For DIOS

concordance calculations, siblings were used as a proxy for DZ twins by correcting the DIOS status for age difference, by using the DIOS status of the older sibling when he/she was the age of the younger sibling. Power calculation was done using DSTPLAN (M. D. Anderson Cancer Center). One set of nonidentical triplets in which 1 member had MI and none had DIOS was counted as a single MI-discordant pair and as a single pair without DIOS. Three families had a set of MZ twins and a sibling all with CF and contributed to both the MZ and sibling pair concordance calculations. With one exception, the MZ twin set was concordant for disease (MI or DIOS) or lack of disease, and it was counted as a single individual within a sibling pair. The exception was an MZ twin pair discordant for DIOS with a sibling without DIOS; this set was counted as a single discordant sibling pair.

Statistical Analysis

Statistical calculations were performed using STATA (StataCorp, College Station, TX). For all tests, a p value <0.05 was considered statistically significant. The Fisher exact test was used when one or more cells had 5 or fewer observations (<http://www.unc.edu/~preacher/fisher/fisher.htm>). Student's t -test was used to evaluate means derived from normally distributed continuous data. Multiple logistic regression was performed with STATA using clinical data from the CF Twin and Sibling Study. Each clinical variable was tested for correlation with MI along with age and gender in a three-variable logistic model. Variance was estimated using the robust Huber/White/sandwich estimator, and observations within each family were not considered to be independent. Factors tested individually for correlation with MI were: age, gender, DIOS, surgery for DIOS, pancreatic insufficiency, number of $\Delta F508$ alleles, sweat chloride, positive cultures for *P.aeruginosa*, mucoid pseudomonas, antibiotic resistant pseudomonas, *B.cepacia*, atypical mycobacteria, aspergillus, *S.aureus*, methicillin-resistant *S.aureus*, *S.maltophilia*, *A.xylosoxidans*, *K.pneumoniae*, *E.coli*, *H.influenzae*, streptococcus, sinus disease, surgery for sinus disease, nasal polyps, surgery for nasal polyps, elevated transaminases, pancreatitis, surgery for pancreatitis, diabetes, cholelithiasis, cholecystectomy, gastroesophageal reflux, Nissen fundoplication, gastrostomy tube placement, rectal prolapse, appendiceal disease, failure to thrive, steatorrhea, neonatal jaundice, average FEV1 (CF-specific percentile score (29)), average BMI Z-score, age at diagnosis of CF, lung transplantation, number of pulmonary exacerbations in the last 1 or last 5 years, and medication compliance. Factors significantly correlated with MI in the three-variable analysis were tested for inclusion in a multivariate logistic regression model. Factors were removed if they did not contribute significantly to the risk of MI and their inclusion did not improve the model (likelihood ratio test), or if they were strongly correlated with another covariate. In the final multivariate model, the 3 parameters regarding number of $\Delta F508$ alleles were correlated (as expected), but no other pair of factors had a correlation coefficient of greater than 0.25.

Genetic Analysis

The genome-wide and chromosome 19-specific STR marker data were screened for mendelian inconsistencies by PedCheck (30) and SIB-PAIR 0.99.9 (31); inconsistent markers were retyped or eliminated. Single- and multipoint, parametric and nonparametric linkage analyses were performed using GENEHUNTER (32) and MERLIN (33). Parametric analyses were performed with dominant, additive, and recessive models of inheritance with penetrance set at 0.80 and phenocopy rate set at 0.10 (both derived from observed rates of MI; see below), and with alternative penetrance values of 0.60 and 1.00 and phenocopy values of 0.00 and 0.20. Analysis using MERLIN included error detection through identification of improbable recombination events. For analysis of high-density SNP data, MERLIN was again used; for this analysis, linkage disequilibrium between markers was modeled by clustering correlated markers (34). For the 7 STR map of chromosome 19, SIB-PAIR 0.99.9 (31) was used to perform identical-by-descent (IBD) analyses. If parental genotype data was not available, then IBD was

estimated from identical-by-state (IBS). ASP 26.07.2001 (35) was used to simulate ($n=1000$) the statistical power of our dataset under a dominant (disease allele frequency = 0.10), recessive (disease allele frequency = 0.43), or additive model (disease allele frequency = 0.10) with the disease gene located at a recombination fraction $\theta = 0.01$ from the simulated marker and a phenocopy rate of 0.05. The above disease allele frequencies were calculated assuming Hardy-Weinberg equilibrium, a trait prevalence of 15% (derived from the observed rate of MI), and a trait penetrance of 80% (derived from the concordance rate in MZ twins). With disease allele frequency defined as q , calculations were as follows: $q^2 \times 0.8 = 0.15$ giving $q = 0.43$ for recessive; $(1 - p^2) \times 0.8 = 0.15$ giving $p = 0.90$ and $q = 0.10$ for dominant; $(2pq + 2q^2) \times 0.8 = 0.15$ giving $q = 0.10$ for additive. For all three models, the number of alleles and the allele frequencies calculated by Genehunter for marker D19S112 were used (13 alleles: 0.0029, 0.1556, 0.0039, 0.0616, 0.0352, 0.2076, 0.2074, 0.0020, 0.1301, 0.1223, 0.0577, 0.0108, 0.0029). The total power of the study was calculated using restricted model estimates and the equation: $[1 - (1 - \text{concordant estimated power})(1 - \text{discordant estimated power})] \times 100\% = \%$ power.

Results

Clinical information collected from 65 pairs of monozygous (MZ) twins, 22 pairs of dizygous (DZ) twins, one set of non-identical triplets, 14 single siblings, 365 sibling pairs (includes 4 sibling-MZ twin pairs), 29 sets of 3 siblings, and one set of 5 siblings (1009 patients total) was obtained from the CF Twin and Sibling Study. All participants meet diagnostic criteria for CF. These patients account for approximately 85% of all twins affected with CF and 35% of all families with two or more children affected with CF in the United States according to the CF Patient Registry maintained by the U.S. CF Foundation. Of the 940 patients with information about MI, 160 had MI (17%); treatment was known in 159 of these: 108 patients had surgical treatment, 49 patients were treated with an enema, 1 patient had an enema and surgery, and 1 resolved spontaneously.

Pancreatic insufficiency and CFTR genotype are correlated with MI

Approximately 95% of CF patients have pancreatic insufficiency (PI), and prior studies of MI have shown a close correlation between pancreatic status and MI (1). Indeed, 159 of the 160 MI patients in this study are diagnosed as PI. As expected, pancreatic sufficiency (PS) was present at a higher rate in patients without MI (91 of 762; 12%; $p=3 \times 10^{-7}$). Since MI correlates with pancreatic status, and pancreatic status correlates with *CFTR* genotype, we expected that MI should also correlate with *CFTR* genotype. As predicted, the common CF mutation $\Delta F508$, which is highly associated with PI, was found at increased frequency in MI vs. non-MI patients (79% vs. 68%; $p=9 \times 10^{-5}$), and homozygosity for $\Delta F508$ was also increased in MI vs. non-MI patients (64% vs. 47%; $p=2 \times 10^{-4}$). Furthermore, of the 39 *CFTR* genotypes found in PS patients in this study, only 1 genotype ($\Delta F508/2184\text{insA}$) also occurred in a patient with MI: the single MI patient who remains PS (complete list of *CFTR* mutations available from corresponding author). Intriguingly, 31 patients with PI carried one mutation observed in PS patients, and none of these patients had MI, whereas among 784 PI patients with no PS mutations, 153 had MI ($p=0.002$). The latter observation suggests that *CFTR* genotype is more predictive of MI than pancreatic status. To test this concept, we selected only PI patients and recalculated the frequency of the $\Delta F508$ mutation and the frequency of homozygosity for $\Delta F508$ in MI vs non-MI patients. Both $\Delta F508$ and homozygosity for $\Delta F508$ were found at greater frequency in MI patients than in non-MI patients ($\Delta F508$ allele frequency: 79% vs. 73%, $p=0.02$; $\Delta F508$ homozygote frequency: 64% vs. 54%, $p=0.01$). Finally, we performed a multivariate regression analysis in which PI was subdivided based on the number of $\Delta F508$ alleles. As shown in Table 1, PI with $\Delta F508$ homozygosity was an independent risk factor for MI (OR 13.0). PI in the context of other *CFTR* genotypes had a large effect size (OR 7.8–8.4)

but was of borderline significance. Thus, while PI is highly correlated with MI, *CFTR* genotype can decrease the association with MI (e.g. PS mutations) or increase association with MI (e.g. $\Delta F508$ homozygosity) in PI patients.

Complications correlated with MI and mode of treatment of MI

To determine if other features of CF were correlated with MI, a series of disease characteristics (listed in Methods) was tested for association with MI by single and multiple logistic regression analysis. In addition to pancreatic status and *CFTR* genotype (shown above), MI was also correlated with distal intestinal obstruction syndrome (DIOS), DIOS treated surgically, elevated transaminases, age, decreased BMI Z-score and *B.cepacia* positive culture (Table 1). We then considered treatment modality (surgical vs. nonsurgical) for MI under the hypothesis that surgical treatment is a marker for more severe intestinal disease. By multiple regression analysis, the only variable significantly correlated with treatment mode for MI was DIOS (odds ratio of having had surgery=2.6; 95% confidence interval 1.1–5.9; $p=0.03$). Further examination of DIOS and MI revealed that the rate of DIOS in patients without MI (96 of 733, 13%) was lower than patients with MI treated surgically (52 of 155, 34%; $p=9\times 10^{-9}$) but about the same in patients with MI treated nonsurgically (10 of 51, 20%; $p=0.2$). Of note, *CFTR* genotype was not correlated with MI treatment modality. No significant correlation between MI or MI treatment modality was observed for gender or other markers for CF disease severity.

Modifier genes contribute substantially to MI

To determine if genetic or nongenetic factors cause MI, we compared the rates of concordance for MI among affected twin pairs and sibling sets, who share both copies of *CFTR* but otherwise have different degrees of gene sharing (Table 2). Among families where at least one member had MI, MZ twins show significantly greater concordance than DZ twins and siblings. Concordance rates for MI did not differ between DZ twins and siblings. In the subjects homozygous for $\Delta F508$, concordance rates were similarly greater among MZ twins (11 of 13 MZ twin pairs; 1 of 5 DZ twin pairs, $p=0.02$; 12 of 44 sibling pairs, $p=0.0003$; 2 of 6 sibling trios, $p=0.05$). It is possible that intestinal obstruction due to MI may affect all CF patients but common protective factors (genetic and otherwise) prevent the development of this complication in most patients. A protective role for modifying factors of MI is suggested by the observation that intestinal obstruction is a consistent rather than uncommon complication in the murine model of CF (15). The concordance rates for the absence of MI are significantly greater in MZ twins than in DZ twins or sibling sets (Table 2). Concordance for lack of MI did not differ between DZ twins and siblings, and results were essentially identical when restricting analysis to homozygous $\Delta F508$ subjects (not shown). Finally, we investigated whether sharing the mode of MI treatment correlated with degree of gene sharing. However, MZ twins did not differ from DZ twins or 2 affected siblings when distributed according to treatment modality (both treated with surgery; one treated with surgery and the other with enema; both treated with enema; data not shown). These data show that MZ twins demonstrate greater concordance in both causative and protective models for MI, suggesting predominance of genetic factors in each model.

Modifier genes do not contribute substantially to DIOS

Since DIOS was highly correlated with surgically treated MI and DIOS, and MI have similar clinical manifestations (8;36), we explored the possibility that they have the same genetic etiology. As compared to MI, the $\Delta F508$ allele of *CFTR* is less well correlated with DIOS. Although the frequency of the $\Delta F508$ allele is greater (76% vs. 70%; $p=0.02$) in subjects with DIOS, the frequency of $\Delta F508$ homozygosity is similar (58% vs. 50%; $p=0.1$). Furthermore, DIOS occurred at similar rates (20%) in subjects homozygous for $\Delta F508$ and in those with other *CFTR* genotypes (15%; $p=0.08$). In striking contrast to MI, the concordance rates for

DIOS (Table 3) are low in MZ twins, and MZ twins and sibling pairs have similar concordance rates ($p=0.5$; Fisher exact) indicating that genetic factors do not play a major role in DIOS. We estimated that these patients provided 80% power for detecting a rate difference greater than 0.25. Concordance rates could not be estimated precisely in DZ twins and 3 affected sibling sets due to small numbers in these groups. Concordance rates for absence of DIOS did not differ among the 2-member groups; the decrease in concordance for lack of DIOS among sets of 3 siblings is no longer present when siblings in each set of three are taken pairwise (concordance rate 75%). DIOS concordance rates for $\Delta F508$ homozygotes were similarly low in all twin and sibling groups (1 of 11 MZ twin pairs; 0 of 3 DZ twin pairs, $p=1$; 7 of 34 sibling pairs, $p=0.7$; 0 of 4 sibling trios, $p=1$). No significant differences were seen in the concordance for lack of DIOS in $\Delta F508$ homozygote twin and sibling groups (not shown). The frequency of DIOS is not significantly different among MZ twins and siblings, while DZ twins have a trend toward a lower rate that likely reflects their younger median age. To assess whether the study population adequately captured the range of ages at which DIOS is usually diagnosed, we compared the mean age at diagnosis of DIOS to mean age of the entire twin or sibling groups. For the MZ twins and the siblings, the mean age at diagnosis of DIOS is lower (12.0 years and 9.0 years, respectively) than the mean age of the MZ twins and siblings (15.5 years and 13.4 years, respectively). Only 5 of 47 DZ twins/triplets were diagnosed with DIOS (average age of 6.4 years at diagnosis), likely reflecting the younger mean age of this population (8.4 years), as previously noted. Unlike MI, these results indicate that modifier genes do not contribute substantially to DIOS, suggesting that DIOS and MI have different etiologies.

Identification of regions that may contain MI modifier genes

The high rate of concordance for MI among MZ twins compared to DZ twins and siblings (all of whom share *CFTR* alleles) indicates that one or more modifier genes exist that cause or protect from MI. To identify regions that may contain MI modifier genes, we performed a genome-wide linkage analysis using short tandem repeat (STR) polymorphisms. Since *CFTR* genotype contributes to MI risk, linkage analysis was also performed on the subset of subjects who were homozygous for the $\Delta F508$ mutation. Forty-six patients with MI who had at least one sibling with MI (2 DZ twin pairs, 18 sibling pairs, and 2 sibling trios; total 26 pairs) and all available parents were genotyped for 402 STR markers with an average spacing of 10 cM. Of these patients, 34 were homozygous for $\Delta F508$ (14 pairs and 2 trios; total 20 pairs). Identity-by-descent (IBD) allele sharing methods were used to identify regions linked to MI. The only region exhibiting significant linkage (logarithm of odds of linkage or LOD >3.0) encompassed the *CFTR* gene on chromosome 7 (multipoint nonparametric LOD score 4.17; see Figure 1A, open circles). Peaks suggestive of linkage (LOD >2.0) were detected on chromosomes 8p23.1 (LOD 2.14) and 11q25 (LOD 2.18) (Figure 1B–C, open circles). When analysis was restricted to $\Delta F508$ homozygotes concordant for MI, the odds of linkage diminished for all three peaks (Table 4). To search for genes that protect CF patients from developing MI, we performed a linkage analysis using 282 pairs (224 nuclear families, 16 trios, and 1 set of 5) concordant for the absence of MI. No regions of linkage with a LOD score greater than 2.0 were found when all *CFTR* genotypes were included (not shown). Because this group without MI includes individuals who may not be susceptible to MI (such as pancreatic sufficient patients), a second analysis was restricted to 128 pairs of $\Delta F508$ homozygotes, all with pancreatic insufficiency (97 nuclear families, 7 trios, and 1 set of 5). Suggestive LOD scores were found on chromosomes 20p11.22 (2.20) and 21q22.3 (2.38); the LOD score for the STR marker nearest to *CFTR* was 11.35 (Figure 2). Thus, multiple loci of suggestive linkage for causative or protective MI modifier genes were found.

To evaluate the regions of possible linkage identified with STR markers and to search for additional regions of linkage, we performed a high-density linkage analysis on the same group of families concordant for the presence of MI (26 affected sibling pairs) using the Affymetrix

Centurion single nucleotide polymorphism (SNP) array. 90,689 SNPs with an average spacing of 34 Kb were genotyped. As expected for a denser marker map, the peak LOD score for the region encompassing the CFTR gene was increased (multipoint nonparametric LOD 6.96), and the region of maximal linkage was narrower (0.6 cM) than in the STR analysis (Figure 1A, solid line). Linkage peaks were again found on chromosomes 8 and 11, though the LOD scores were lower (Figure 1B–C, solid lines; Table 4). The only region with LOD near 2.0 in the SNP analysis was on chromosome 4q35.1 (nonparametric LOD 1.96; Figure 1D, solid line), a region whose nearest marker in the previous 10 cM STR map had a LOD score of 1.62. In analysis restricted to the 20 pairs of MI-concordant $\Delta F508$ homozygotes, LOD scores for CFTR on chromosome 7 and for loci on chromosome 8 and 11 decreased (Table 4), while the peak LOD on chromosome 4 increased to 2.42 (Figure 1D, dashed line). Thus, the SNP map improved sensitivity and resolution for detecting CFTR linkage on chromosome 7 and provided evidence of linkage to MI, in $\Delta F508$ homozygotes, on chromosome 4q35.1.

MI is not linked to the CFM-1 locus on chromosome 19q13

Meconium ileus had been previously linked to the CFM1 region on chromosome 19q13, but our genome-wide analyses with low- and high-density marker maps did not reveal a linkage peak on this chromosome (Figure 3A). To provide a direct comparison with previously used STR markers, we typed 7 STR markers spanning 6.5 Mb of the CFM1 region in a subset of the above group, including 24 pairs concordant for the presence of MI, 65 discordant pairs, and 130 pairs without MI. Analyses were performed on the entire group and on those concordant for MI or lack of MI. Five of the 7 markers were the same as those used in the study by Zielenski et al. (D19S219, D19S112, D9S412, D19S902, and D19S604; Figure 3, solid diamonds). The highest nonparametric LOD score was 0.14 for all pairs (0.42 in MI concordant pairs). Under recessive, dominant and additive models of inheritance, LOD scores never exceeded 0.00 (0.06 in the concordant pairs) and the heterogeneous LOD scores never exceeded 0.25 (0.08 in the concordant pairs). Analysis to evaluate linkage to a protective allele on chromosome 19q13 produced a LOD score of 0.42 (0.67 in pairs concordant for the absence of MI and 1.18 when restricted to $\Delta F508$ homozygotes). As a second means of assessing linkage, haplotypes were manually constructed based on identity-by-state from markers D19S217, D19S219, and D19S112 for the siblings concordant for MI, discordant for MI, or unaffected with MI. The proportion of siblings sharing 0, 1, or 2 haplotypes across the region did not deviate from the expected distribution of 25%, 50% and 25%, respectively ($p=1$, χ^2). Finally, to evaluate for genetic heterogeneity, 5 sets of concordant siblings that did not share at least one haplotype and 14 sets of discordant siblings sharing both haplotypes were excluded, and the linkage analysis was repeated. Without these families, the highest LOD score was 0.40 (1.74 in the concordant pairs). Thus, we found no evidence for linkage of MI to the CFM1 locus in this sample of CF twins and siblings.

Discussion

Cystic fibrosis (CF), like many other “single gene” disorders, displays considerable variability that cannot be attributed to allelic differences in the disease-causing gene (3). Meconium ileus is a well-recognized complication of CF that has been a paradigm for the elucidation of modifying factors (37). Prior studies of CF siblings documented that familial recurrence of MI exceeded the incidence of MI in unrelated patients (4;11–13;17). However, siblings not only share 50% of their genes but they also have similar environmental exposures (e.g. parents, home, clinic). The significantly higher concordance rate of MI in affected MZ twins that share 100% of their genes, compared to affected DZ twins who share 50% of their genes, indicates that genetic factors are likely to play a substantial role in MI. While acknowledging that the number of study subjects is small, we can estimate that the contribution of genetic variation (heritability or h^2) to developing MI approaches 1.0 ($h^2 = 2(\text{MZ concordance} - \text{DZ}$

concordance) (28)). A more robust estimate which considers the disease prevalence (27) is required to estimate heritability for absence of MI (which “occurs” in ~85% of the CF population). Heritability estimates for lack of MI also approach 1.0 (0.80 to 1.4 as estimated by DZ and sibling pairs, respectively). Thus, there is evidence for the presence of both protective and susceptibility modifier genes for MI.

Comparison of the recurrence rates of MI among twins and siblings facilitates predictions of the penetrance, number and inheritance pattern of the modifier gene(s). The high rate of concordance in the MZ twins suggests that penetrance of genetic factors is high (e.g. >80%). In the 2 affected sibling sets, 19 pairs were concordant for MI and 61 pairs were discordant indicating a sibling recurrence rate of 0.24. Within the ten sets of 3 affected siblings with MI (2 concordant sets and 8 discordant sets in Table 3), there are 30 possible pairwise combinations; nine pairs are concordant and 16 pairs are discordant for MI giving a sibling recurrence rate of 0.36. These estimates compare favorably with recurrence rates that can be derived from families with 2 or more siblings affected with CF provided by Donnison et al (Concordant 16/Discordant 54; 0.23; (11)), Allan et al (Concordant 7/Discordant 16; 0.3; (12)), Kerem et al (Concordant 5/Discordant 15; 0.25;(4)), Zielenski et al (Concordant 7/Discordant 33; 0.18; (17)) and Picard et al (Concordant 10/Discordant 40; 0.2;(13)). A recurrence risk of 0.24 among siblings is similar to that observed for single gene recessive disorders such as CF. The results of the genome-wide linkage analyses indicate that a single modifier gene is unlikely to account for MI. Thus, we postulate that two or more modifier genes of relatively high penetrance are primarily responsible for MI.

While the classic twin study has proven to be a reliable means of distinguishing the effects of shared environment from shared genes, potential limitations have been discussed (38). For example, although it is commonly agreed that MZ and DZ twins have similar degrees of shared environment, there have been examples where MZ twins have a unique shared environment (e.g. placenta) causing elevated concordance rates in MZ twins leading to an inflated estimate of heritability (38). At this point, we cannot exclude this possibility. Furthermore, the collection of DZ twins in this study has an excess of males, indicating an incomplete ascertainment of this group, so the concordance rates in DZ twins may be different than calculated. This could alter our heritability estimates, possibly increasing the predicted role for non-genetic factors. The presence of three sets of MZ twins discordant for MI in this study and the published report of an MZ pair discordant for MI (39) indicate that non-heritable factor(s) play a role in MI. Nevertheless, the above findings, particularly the high rate of concordance in MZ twins, support the conclusion that the risk for MI is primarily inherited.

Although MI has been reported in a few patients that do not manifest CF (40;41), MI in CF patients has almost always been reported in the context of pancreatic insufficiency (PI) (1). Exocrine pancreatic function in CF patients depends on the underlying mutations in *CFTR*, with mutations resulting in loss of *CFTR* function being almost invariably associated with PI. While most of the patients with MI in this study had PI, we discovered that the frequency of MI in PI patients differed depending upon *CFTR* genotype. These results suggest that the *CFTR* genotype affects the risk for MI beyond its role in determining pancreatic status. This conclusion is consistent with prior studies of unrelated CF patients. CF patients bearing the G551D mutation have been shown to have a lower rate of MI than those homozygous for $\Delta F508$ even though both mutations are almost invariably associated with PI (42;43). Thus, MI appears to be the consequence of interaction between the *CFTR* gene and MI modifier genes.

Cross-sectional and longitudinal studies indicate that CF patients with MI follow a different course than those without MI (6;44;45). Some have suggested that complications arising from surgical treatment of MI are the primary reason for this difference (46). To determine if the effect of MI modifier genes extends to other manifestations of CF, we performed multivariate

regression analysis to identify significant covariates of MI. In addition to pancreatic status and *CFTR* genotype, we found that MI was highly correlated with several other gastrointestinal manifestations of CF including elevated liver enzyme levels, DIOS, and reduced BMI. An inverse correlation with increasing age suggests that the strict criteria for diagnosis of MI in the current study are more difficult to fulfill in older patients (for whom neonatal records are less accessible, for instance); alternatively, this could reflect a shorter life expectancy for MI patients as has been reported (6). Although MI was associated with *B. cepacia* infection, we were unable to correlate MI with any other markers of pulmonary disease severity. MI has been previously associated with liver disease (47), poorer nutritional outcomes (48), and DIOS (46) though correlation with mode of MI treatment was not specifically addressed in these studies. The clinical and pathological similarities between DIOS and MI have suggested a common etiology (8;9;46). However, our concordance analysis suggests that MI and DIOS are caused by different modifying factors. At present, the complexity of covariate analysis is limited by the number of patients in the study; more exhaustive evaluation of possible covariates of MI and of DIOS will be performed when recruitment for the CF Twin and Sibling Study is completed.

Genome-wide linkage analyses did not identify a single major locus for MI, although we had ample power as indicated by the significant LOD scores obtained for the region encompassing *CFTR*. Instead, multiple loci with LOD scores suggestive of linkage were identified, in agreement with expectations from sibling recurrence risks (above). While it is clear that the data support a polygenic origin for MI, further studies are required to determine whether the identified loci contain modifier genes for MI. Of note, the peak on chromosome 11q25 is in a region of conserved synteny with a segment of mouse chromosome 9 associated with intestinal obstruction in a CF mouse model (49). Two additional points are worth noting. First, the high density map composed of SNP markers localized *CFTR* on chromosome 7 with a substantially higher LOD score and better defined peak of linkage than the coarse map composed of STR markers. This pattern is expected for a linkage region containing a *bona fide* trait causing gene. Although the high density map showed lower odds of linkage than the STR map for chromosomes 8p23.1 and 11q25, it did increase for a region on chromosome 4 that had a LOD score in the coarse STR map that did not exceed the threshold of suggestive linkage ($\text{LOD} \geq 2.0$) (50). The LOD score for chromosome 4q35.1 was further enhanced when linkage analysis was restricted to $\Delta F508$ homozygotes despite a reduction in the number of pairs analyzed from 26 to 20. The latter behavior is consistent with evidence that *CFTR* genotype influences risk of developing MI. Under this circumstance, one would predict that restricting linkage analysis to patients bearing high-risk *CFTR* genotypes should optimize the power to detect MI modifier genes. Second, we were unable to detect the previously reported linkage of MI to a locus on chromosome 19q13 (17). The power to detect linkage to a single locus in the previous study at an alpha value of 0.05 was 68%, 35%, and 58% under a recessive, additive, or dominant model, respectively. The power of this study to detect linkage at the same alpha value was 92% under a recessive model; 56% under an additive model and 80% under a dominant model. Furthermore, removal of unambiguously unlinked sibling pairs from the analysis did not expose linkage disguised by locus heterogeneity. There are several possible reasons for the difference between our result and that of Zielenski and colleagues. Meconium ileus may be the consequence of multiple modifier genes as suggested by studies of the CF mouse (16;49) and the results of the linkage scans presented here. Differences in the stratification of the sibling populations including differences in *CFTR* genotype prevalence may have exposed a modifier gene of minor effect on chromosome 19 in the initial study but not in the current study. Finally, the diagnosis of MI involves subjective criteria that may have led to differences in the phenotyping of the MI populations in each study.

In summary, we have used the classic twin design to estimate the contribution of genetic and non-genetic modifiers to a well-known recognized complication of CF. Demonstration that

genetic modifiers contribute substantially to MI justifies efforts to identify MI modifier genes by virtue of their location. Evidence that *CFTR* genotype contributes to MI risk emphasizes the importance of considering this variable in future modifier studies of MI. Our results indicating that MI is polygenic will inform the design of future searches for modifier genes responsible for this intestinal complication.

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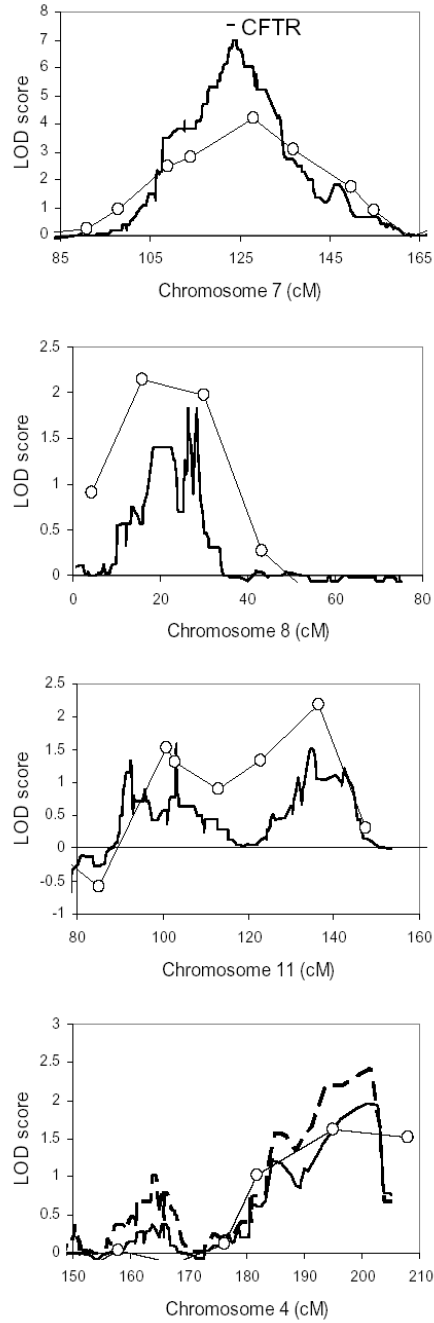


Figure 1. Linkage peaks identified in 26 CF sibling pairs concordant for presence of MI
Shown are multipoint nonparametric LOD scores for linkage using the STR map (open circles) and SNP map (solid line) in 26 pairs with all CFTR genotypes included. The CFTR gene location on chromosome 7 is marked by the bar. For chromosome 4, also shown are linkage results from SNP genotyping in the subset of 20 MI-concordant pairs homozygous for $\Delta F508$ (dashed line). For comparison of peak widths, 80 cM width is shown for each chromosome.

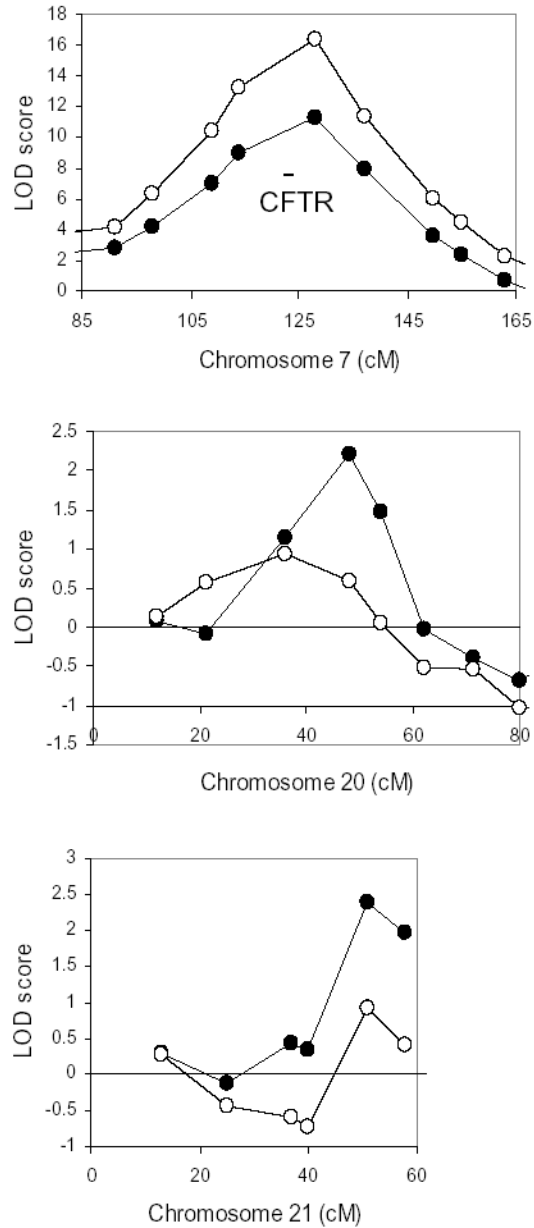


Figure 2. Linkage peaks identified in patients concordant for the absence of MI

Shown are multipoint nonparametric LOD scores for linkage using the STR map in 282 pairs with all CFTR genotypes included (open circles) and in the subset of 128 pairs without MI and homozygous for $\Delta F508$ (filled circles). The CFTR gene location on chromosome 7 is marked by the bar. For comparison of peak widths, 80 cM width is shown for each chromosome.

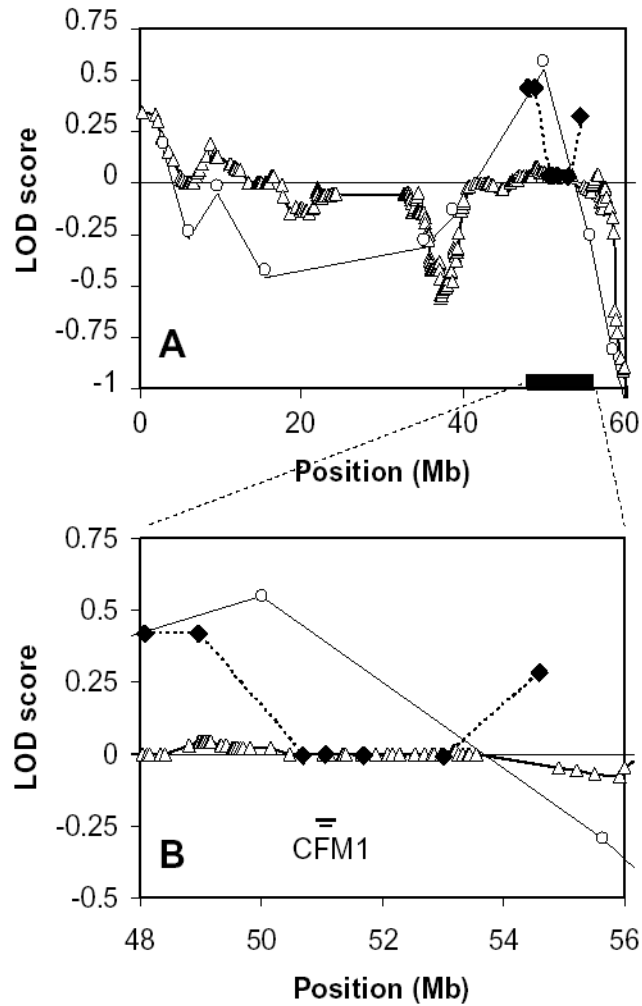


Figure 3. Linkage analysis of the chromosome 19q13 region encompassing the CFM1 locus (A) Entire chromosome 19, (B) the CFM1 region at chromosome 19q13. Shown are linkage results from the genome-wide STR map (open circles), the genome-wide SNP map (open triangles), and the chromosome 19-specific map of 7 STR markers (diamonds) which includes 5 STR markers used in a prior study of MI (17) and 2 additional STR markers (diamonds with asterisks). The region from 48–56 Mb encompasses 2 of the Marshfield STR markers, the 7 STR marker map, and 165 of the SNP markers. The location of CFM1 is indicated by the bar.

Table 1

Multivariate regression analysis of MI covariates

	Odds Ratio	95% Conf. Interval	P value
Pancreatic insufficiency with no $\Delta F508$ alleles	8.4	(0.9, 77.2)	0.061
Pancreatic insufficiency with one $\Delta F508$ allele	7.8	(1.0, 61.2)	0.050
Pancreatic insufficiency with two $\Delta F508$ alleles	13.0	(1.7, 100)	0.014
DIOS ^a treated nonsurgically	2.52	(1.47, 4.32)	0.001
DIOS treated with surgery	24.6	(8.34, 72.6)	7×10^{-9}
Elevated ALT/AST ^b	4.15	(2.25, 7.67)	5×10^{-6}
Current Age ^c	0.93	(0.89, 0.97)	0.001
BMI Z-score ^d	1.34	(1.03, 1.75)	0.032
<i>B. cepacia</i> positive culture	3.33	(1.48, 7.47)	0.004

^aDistal intestinal obstruction syndrome

^bAlanine aminotransferase (ALT) or aspartate aminotransferase (AST) levels greater than twice normal upper limit at least twice

^cOdds ratio reflects decreased odds of MI for each year of age.

^dOdds ratio reflects increased odds of MI for each unit decrease in BMI Z-score.

Table 2

MI Status of Twins and Siblings

	MZ twins	DZ twins	2 affected siblings	3 affected siblings
Total # of Families	65	23	326	27
# Sets MI Concordant	14	2	19	2
# Sets MI Discordant	3	7	61	8 ^a
# Sets without MI	48	14	246	17
Concordance for MI	82%	22% ^b	24% ^c	20% ^d
Concordance for no MI	94%	67% ^e	80% ^f	68% ^g

^aTwo families had 2 affected children with MI; 5 families had one affected child with MI.

^bp=0.009 vs. MZ twins

^cp= 1×10^{-5} vs. MZ twins

^dp=0.003 vs. MZ twins

^ep=0.005 vs. MZ twins

^fp=0.02 vs. MZ twins

^gp=0.04 vs. MZ twins

Table 3

DIOS status of twins and siblings

	MZ twins	DZ twins	2 affected siblings ^a	3 affected siblings ^a
Total # of families	60	23	312	25
# sets DIOS concordant	5	0	16	0
# sets DIOS discordant	13	5	56	12 ^b
# sets without DIOS	42	18	240	13
Concordance for DIOS	28%	0%	22%	0%
Concordance for no DIOS	76%	78%	81%	52%

^aSiblings were corrected for age difference by determining the DIOS status of each sibling at the age of the youngest sibling in the sibship.

^bIncludes 9 sets of three siblings in which one member had DIOS and 3 sets in which two members had DIOS.

Table 4A

Multipoint nonparametric LOD scores exceeding 2.0 from genome-wide STR and SNP linkage analysis of patients concordant for presence of MI.

Chromosome	cM (Marshfield)	STR markers All (n=26)	$\Delta F508/\Delta F508$ (n=20)	cM (deCODE)	SNP markers All (n=26)	$\Delta F508/\Delta F508$ (n=20)
7 (CFTR)	128	4.17	3.42	123.559–124.152	6.96	5.09
8p23.1	16	2.14	2.07	18.065–22.332	1.40	0.90
11q25	136.5	2.18	1.94	134.172–135.623	1.52	1.76
4q35.1	195.06	1.62	1.71	193.7–201.4	1.96	2.42

NOTE: Analyses are shown from all CFTR genotypes (All) and $\Delta F508$ homozygotes ($\Delta F508/\Delta F508$); n refers to the number of pairs.

Table 4B

Multipoint nonparametric LOD scores exceeding 2.0 from genome-wide STR linkage analysis of patients concordant for absence of MI.

Chromosome	cM (Marshfield)	STR markers All (n=282)	$\Delta F508/\Delta F508$ (n=128)
7 (CFTR)	128	16.4	11.4
20p11.22	48	0.58	2.20
21q22.3	51	0.93	2.38