Variants in the Glutamate-Cysteine-Ligase Gene Are Associated with Cystic Fibrosis Lung Disease

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Background: Chronic progressive lung disease is the most serious complication of cystic fibrosis (CF). Glutathione plays an important role in the protection of the CF lung against oxidant-induced lung injury.

Objectives: We hypothesized that a polymorphism in a novel candidate gene that regulates glutathione synthesis might influence CF lung disease.

Methods: In a cross-sectional study, subjects were recruited from CF clinics in Seattle and multiple centers in Canada. We tested for an association between CF lung disease and a functional polymorphism in the glutamate-cysteine ligase catalytic subunit (*GCLC*) gene. Multiple linear regression was used to test for association between polymorphisms of *GCLC* and severity of CF lung disease while adjusting for age, *Pseudomonas aeruginosa* infection, and cystic fibrosis transmembrane conductance regulator (*CFTR*) genotype. Analysis was repeated for patients with CF stratified by *CFTR* genotype.

Measurements and Main Results: A total of 440 subjects with CF participated in the study (51% male; mean $\pm$ SD age, 26 ± 11 yr; mean FEV₁, $62 \pm 28\%$ predicted). In the total population, there was a trend toward an association between *GCLC* genotypes and CF lung disease (linear regression coefficient [SEM], 1.68 [1.0]; $p = 0.097$). In the stratified analysis, there was a highly significant association between *GCLC* genotype and CF lung function in subjects with a milder *CFTR* genotype (linear regression coefficient [SEM], 5.5 (1.7); $p = 0.001$). **Conclusions:** In patients with CF with a milder *CFTR* genotype, there is a strong association between functional polymorphisms of the *GCLC* gene and CF lung disease severity.

Keywords: CFTR genotype; glutathione; modifier genes

Cystic fibrosis (CF) is an autosomal recessive genetic condition that is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene (1). CF is characterized by the clinical phenotype of recurrent respiratory infection, pancreatic insufficiency, and elevated sweat chloride. Median survival is into the mid-thirties, with over 90% of patients dying of progressive respiratory failure (2).

The clinical course in CF is highly variable and is not fully explained by mutations in the *CFTR* gene (3–5). Family-based studies have suggested that host genetic factors that code outside of *CFTR* may modify the CF phenotype (6, 7). Identifying modifier genes that influence the development of lung disease is of primary importance in understanding the pathophysiology of CF lung disease and in identifying new therapeutic strategies to slow the progression of disease (8, 9).

Glutathione (GSH) is a ubiquitous tripeptide that plays a crucial role in the protection of the lung from oxidant-induced lung damage (10). Oxidant-induced lung injury is an important mechanism of lung damage in CF (11–14). Patients with CF have low levels of GSH in the epithelial lining fluid (15), suggesting that deficiency in GSH may influence the development of CF lung disease. We hypothesized that functional polymorphisms in a novel candidate gene that regulates GSH synthesis may modify the severity of lung disease in patients with CF.

The rate-limiting step in the production of GSH is the formation of γ -glutamylcysteine, catalyzed by glutamate-cysteine ligase (GCL) (16). GCL is composed of two subunits, a catalytic subunit (*GCLC*) and a modifier subunit (*GCLM*) (17, 18). The 5'-untranslated region of *GCLC* mRNA (NM_001498) has been shown to contain a polymorphic guanine-adenine-guanine (GAG) trinucleotide repeat (TNR) just upstream of the translation start codon (19). This GAG TNR polymorphism is associated with variable levels of GSH in human cells lines, with 7 GAG TNR associated with the lowest GSH levels, 8 GAG TNR associated with intermediate GSH levels, and 9 GAG TNR associated with the highest GSH levels (20).

We tested whether *GCLC* genotypes are associated with more severe CF lung disease. In addition, because certain *CFTR* genotypes are associated with a milder clinical phenotype (3, 5, 21–25) and GSH secretion into the epithelial lining fluid may be influenced by *CFTR* function (26–29), we hypothesized that this effect would be influenced by *CFTR* genotype.

Some of the results presented in the manuscript have been presented in abstract form (30).

METHODS

In a pilot study performed between 1999 and 2001, patients were recruited from the CF Clinic at the University of Washington, Seattle, WA. Based on interesting early results (30), the study population was subsequently expanded to include patients with CF recruited from Canadian CF Clinics in Vancouver, Victoria, Montreal, and Hamilton. All patients were diagnosed with CF based on established criteria (31).

The study design was a cross-sectional study testing for an association between *GCLC* GAG TNR genotypes and severe CF lung disease. At time of entry into the study, the following clinical variables were collected on each subject using chart review: FEV₁(% predicted), age, sex, presence or absence of *Pseudomonas aeruginosa* infection, pancreatic insufficiency status, and *CFTR* genotype. The best FEV₁(% predicted) in the year before recruitment was used to define the pulmonary phenotype. *P. aeruginosa* infection was defined as present if the patient had at least one positive sputum sample in the year before recruitment.

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Pancreatic insufficiency was defined as present based on the need for pancreatic enzyme supplementation.

CFTR genotypes were classified as "severe" and "mild" based on the effects of their *CFTR* mutations on *CFTR* production and clinical phenotype (5). Using the functional class system proposed by Tsui (32) and by Welsh and Smith (33), patients with both mutant alleles in Class I, Class II, or Class III were grouped together as a severe *CFTR* genotype, and patients with at least one mutant allele in Class IV, V, and Unclassified were considered a mild *CFTR* genotype. This classification system has been shown to be associated with significant differences in lung disease severity, with *CFTR* genotypes from Class I–III being associated with worse lung function than those with a Class IV, V, or Unclassified genotype (5).

Microsatellite analysis of the *GCLC* TNR polymorphism is presented in the online supplement.

Statistical Analysis

Multiple linear regression was used to test the relationship between lung disease severity (outcome variable) and the *GCLC* genotypes (exposure variable). Severity of lung disease was estimated using the FEV₁(% predicted) as a continuous variable. The predictor variable, *GCLC* genotype, was grouped as an ordinal variable according to the estimated effect of each *GCLC* genotype on glutathione production (20) (7/7 genotype < 7/8 genotype < 7/9 genotype < Other genotype). *GCLC* genotypes 8/8, 8/9, and 9/9 genotypes were grouped together as "other" due to the rarity of each genotype and the evidence that these repeats are associated with increased GSH production. All other clinical variables (including higher-order terms) were tested for association with lung disease severity. Variables associated with lung disease on univariate analysis ($p < 0.2$) were included in a multivariate model as possible confounders or effect modifiers. The analysis was repeated in patients stratified by *CFTR* genotype grouped as described previously. Postregression diagnostics were performed on the multiple regression model to ensure that all linear regression assumptions were valid. Results were expressed as regression coefficients with standard errors. Two-sided p value < 0.05 was considered statistically significant. All analysis was performed using Stata 8.0 (Stata Corporation, College Station, TX).

RESULTS

Clinical characteristics of the total patient population and the Seattle and Canadian cohorts are shown in Table 1. Clinical and genetic data were available on a total of 440 patients with CF. The mean age of patients was 26.2 yr (range, 5–62 yr). Mean FEV₁(% predicted) was 62% (range, 11–128%). *GCLC* GAG TNR genotype frequencies are shown in Table 2 and were similar to that observed in the white population (20). *GCLC* GAG TNR genotype frequencies were in Hardy-Weinberg equilibrium.

Age, age squared (age²), the presence of *P. aeruginosa* infection, and *CFTR* genotype (severe versus mild) were associated with FEV₁(% predicted) on univariate analysis ($p < 0.2$). On multivariate analysis, there was a trend toward an association

between *GCLC* TNR polymorphism and FEV₁(% predicted) after adjusting for age, age², *P. aeruginosa* infection, and *CFTR* genotype (linear regression coefficient [SEM], 1.68 [1.0]; $p = 0.097$). In the subgroup with a mild *CFTR* genotype, there was a highly statistically significant association between *GCLC* TNR polymorphisms and FEV₁(% predicted) (linear regression coefficient [SEM], 5.5 [1.7]; $p = 0.001$), explaining 4.3% of the variation in FEV₁(% predicted). There was no association between *GCLC* TNR polymorphisms and CF lung disease in patients with a severe *CFTR* genotype (Table 3). Graphical interpretation of the multiple linear regression models demonstrating the effect of *GCLC* genotype on FEV₁(% predicted) is shown in Figure 1.

Internal Validation of Results

The decision to expand recruitment was based on pilot data found in the Seattle cohort. (30) This pilot study found a significant association between *GCLC* polymorphisms and CF lung disease in patients with a milder *CFTR* mutation (linear regression coefficient [SEM], 13.4 [2.8]; $p < 0.001$). To determine the replication validity of our pooled findings, we performed post-hoc analysis of the Canadian patients with CF with a mild *CFTR* genotype separately. The association was not as strong as that observed in the Seattle cohort but was in the same direction and approached statistical significance (linear regression coefficient [SEM], 3.93 [2.14]; $p = 0.069$).

DISCUSSION

The main findings of this study are that functional polymorphisms of the *GCLC* gene are associated with CF lung disease and that this effect is influenced by *CFTR* genotype. These observations are consistent with the known effect of *GCLC* polymorphisms on gene product and the biological role of GSH and *CFTR* in CF lung disease. In addition, the strength of association is large and highly statistically significant. We believe that this supplies compelling evidence that the *GCLC* gene is a genetic modifier of CF lung disease.

Patients with CF demonstrate substantial phenotypic variability (4, 5). Since the discovery of the *CFTR* gene that causes CF, over 1,000 mutations have been identified (34), yet only a small number of *CFTR* genotypes are associated with a milder clinical phenotype (3, 5, 21–25). The effect of *CFTR* genotype on phenotype seems to relate to the functional effects of *CFTR* genotypes on *CFTR* production with very low levels of *CFTR* associated with a severe phenotype and intermediate levels of *CFTR* associated with milder CF (25, 35, 36). Despite this, there is evidence that the clinical course in patients with the same *CFTR* genotype is highly variable, and family-based studies have suggested that genetic factors that code outside of *CFTR* may modify the CF

TABLE 1. CLINICAL CHARACTERISTICS OF SUBJECTS WITH CYSTIC FIBROSIS

	Total Population	Seattle Cohort	Canadian Cohort
N	440	98	342
Sex, % male	51	51	50
CF genotype (severe vs. mild)*	70%	71%	68%
Age, yr	26 ± 11	33 ± 10	23 ± 11
FEV ₁ (% predicted)	62 ± 28	58 ± 24	64 ± 28
Pancreatic insufficiency, %	90	90	90
<i>P. aeruginosa</i> infection, %	66	77	64

Definition of abbreviation: CF = cystic fibrosis.

* Severe cystic fibrosis transmembrane conductance regulator (*CFTR*) mutations (Class I–III) = G542X, R553X, W1282X, R1162X, 621–1G→T, 1717–1G→A, 1078ΔT, 3659ΔC, ΔF508, ΔI507, N1303K, S549N, G551D, R560T. Mild *CFTR* mutations (Class IV and V) = R117H, R334W, G85E, R347P, 3849+10KbC→T, 2789+5G→A, A455E. Other unclassified mutations were also grouped as mild.

TABLE 2. GENOTYPE FREQUENCIES OF GLUTAMATE-CYSTEINE LIGASE CATALYTIC SUBUNIT GAG TRINUCLEOTIDE REPEATS

GCLC Genotype*	Frequency (%)
7/7 allele pair	40
7/8 allele pair	17
7/9 allele pair	28
Other	15

Definition of abbreviation: GCLC = glutamate-cysteine ligase catalytic subunit.

* GCLC genotypes were in Hardy Weinberg Equilibrium ($p = 0.73$).

phenotype (6, 7). A number of possible CF modifier genes have been identified (37–44), although a recent large, multicentered study found that, with the exception of variants in the TGF- β coding region, many of these previously identified modifier genes were not associated with the CF phenotype in over 800 $\Delta F508$ homozygous patients (45).

Genes that influence the GSH pathway have been examined as potential modifiers of the CF phenotype, with polymorphisms in the glutathione-S-transferases M1, M3, and P1 being associated with a more severe CF phenotype (37, 43, 46). This is the first study to describe an association between a novel candidate gene that regulates GSH production and CF lung disease severity. These findings are supported by considerable evidence that GSH plays an important role in the pathophysiology of CF lung disease. Lung inflammation in patients with CF is characterized by an increase in oxidant production from activated neutrophils and macrophages on the respiratory epithelial surface (47–49). This increase in oxidant production is thought to be one of the principal mechanisms through which the lung epithelium becomes damaged in CF (11–14). In the healthy lung, one of the first lines of defense against oxidant stress is the extracellular antioxidant GSH, which is abundantly present in the epithelial lining fluid (ELF). In the CF lung, ELF levels of reduced and oxidized GSH are low, suggesting that in patients with CF, a relative deficiency of GSH is present (15). The reason for this seems to be related to the direct or indirect effects of CFTR on GSH transport out of epithelial cells. CFTR may be an important regulator of GSH release into the ELF (29), with deficiency in CFTR associated with lower levels of GSH (26, 27) and restoration of chloride channel function leading to increased GSH secretion (28).

Our observation that GCLC TNR GAG polymorphisms have a significant influence on CF lung function only in patients with a mild CFTR genotype is consistent with the molecular biology of how CFTR influences the CF phenotype and GSH transport into the ELF. Patients with a severe CFTR genotype have very low levels (< 3%) of functioning CFTR protein, which leads to a more severe CF phenotype. Patients with a mild CFTR genotype have a milder clinical phenotype that is most likely due to the

Figure 1. Fitted regression line with adjusted FEV₁ (% predicted) for each glutamate-cysteine ligase catalytic subunit genotype, stratified by cystic fibrosis transmembrane conductance regulator genotype.

expression of increased amounts (5–13%) of functioning CFTR protein (25, 35, 36). Because CFTR influences the transport of GSH into the ELF, patients with CF with GCLC variants associated with greater GSH production, in the setting of more functioning CFTR, should have higher levels of GSH in the ELF and enhanced protection against oxidant-induced lung injury. Conversely, the severe reduction in functional CFTR observed in patients with CF with a severe CFTR genotype would result in low levels of GSH in the ELF irrespective of the effect of GCLC GAG TNR polymorphisms on GSH synthesis, which is consistent with our findings.

TABLE 3. ASSOCIATION BETWEEN GLUTAMATE-CYSTEINE LIGASE CATALYTIC SUBUNIT GENOTYPE AND CYSTIC FIBROSIS LUNG FUNCTION (n = 440)

Study Population	Adjusted GCLC Genotype Regression Coefficient (SE)*	p Value
All patients (n = 440)	1.68 (1.0)	0.097
Patients with a severe CFTR genotype (n = 301)	0.77 (1.24)	0.533
Patients with a mild CFTR genotype (n = 139)	5.5 (1.7)	0.001

Definition of abbreviations: CFTR = cystic fibrosis transmembrane conductance regulator; GCLC = glutamate-cysteine ligase catalytic subunit.

* GCLC genotype is an ordinal variable with 1 = 7/7, 2 = 7/8, 3 = 7/9, 4 = Other/Other. Patients with at least one mutant allele in Class IV, V and Unclassified were considered to have a mild CFTR genotype.

Although the results of this study require confirmation at a molecular level, these findings may have important therapeutic implications. Our findings show that lung disease severity in patients with CF is influenced by factors that regulate lung GSH levels. It is therefore plausible that therapeutic interventions that increase lung GSH may reduce the decline in lung function seen in CF. Clinical trials that examine the role of supplemental GSH (or GSH precursors) in CF should consider stratification of enrolled patients by *CFTR* and *GCLC* genotype because the therapeutic effects may vary across different genetic subgroups. Our data also suggest that systemic therapies that increase epithelial cell GSH production could be of benefit to patients with CF with a mild *CFTR* genotype. For patients with CF with a severe *CFTR* genotype, our results suggest that systemic therapies that increase epithelial cell GSH levels may not be as beneficial, although inhaled therapy may be a more appropriate method of increasing GSH levels in the ELF of these patients (50).

There are a number of limitations to our study. First, false-positive genetic association studies may occur as a result of chance, population stratification, or the presence of linkage disequilibrium between the allele of interest and the true disease-modifying allele. It is unlikely that chance or population stratification would fully explain our findings, given the strength of the observed association and the replication of findings in different centers (51). We are currently examining for linkage disequilibrium between *GCLC* TNR GAG repeats and other variants in the region of the *GCLC* gene, although, because the nearest gene (NM_021814) is > 140 kb downstream of the *GCLC* gene (52), it is likely that the *GCLC* gene is the true modifier gene. Second, we assumed a linear dose-response relationship between *GCLC* GAG TNR genotype and CF lung function. This is based on earlier data that suggest that the *GCLC* 7 GAG TNR polymorphisms result in less GSH compared with the 8 GAG TNR and 9 GAG TNR (20). This has yet to be confirmed in patients with CF. Ongoing studies in our laboratory are examining *GCLC* mRNA and protein levels to fully understand the differences in transcriptional efficiency, message stability, or translational efficiency associated with *GCLC* GAG TNR alleles.

Another concern is our method of classifying *CFTR* genotype. This was done *a priori*, with *CFTR* mutations associated with severely reduced *CFTR* production (members of the functional Class I, II, and III) grouped as "severe" and those that are likely to have more *CFTR* function (members of functional Class IV, V, and Unclassified) grouped as "mild." We believe this classification method is justified based on the knowledge that *CFTR* influences GSH transport and the substantial evidence that the *CFTR* genotypes we classified as mild are associated with a milder pulmonary phenotype (3, 5, 21–25).

Finally, our analysis was based on the hypothesis that low levels of *CFTR* leads to reduced GSH in the ELF, but there are alternative explanations. Recent studies suggest that *CFTR* expression may be reduced in the setting of increased oxidant stress (53, 54). Because a moderate amount of *CFTR* expression is associated with a milder CF phenotype (36), genetic variation in *GCLC* that leads to improved intracellular oxidant defense may lead to a milder CF phenotype as a result of increased *CFTR* expression. Alternatively, severe *CFTR* genotypes leading to worse clinical disease may result in lower GSH as a result of increased lung inflammation and impaired nutrition. Further studies are necessary to understand the interaction between *GCLC*, GSH, and *CFTR* as modifiers of the CF phenotype.

In conclusion, this study found an association between variants in *GCLC*, a novel candidate gene that influences GSH production, and CF lung function. This effect was observed only in patients with a mild *CFTR* genotype. This is of clinical interest

in terms of predicting which patients with CF will develop more severe lung disease and the potential role the *GCLC* genotype may play in the pharmacogenetics of GSH replacement therapy.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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