Published in final edited form as:

Clin Genet. 2012 December ; 82(6): 546–551. doi:10.1111/j.1399-0004.2011.01804.x.

Cystic fibrosis mutations for p.F508del compound heterozygotes predict sweat chloride levels and pancreatic sufficiency

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Abstract

Cystic fibrosis (CF) is a monogenetic disease with a complex phenotype. Over 1500 mutations in the *CFTR* gene have been identified; however, the p.F508del mutation is most common. There has been limited correlation between the *CFTR* mutation genotype and the disease phenotypes. We evaluated the non-p.F508del mutation of 108 p.F508del compound heterozygotes using the biological classification method, Grantham and Sorting Intolerant from Tolerant (SIFT) scores to assess whether these scoring systems correlated with sweat chloride levels, pancreatic sufficiency, predicted FEV₁, and risk of infection with *Pseudomonas aeruginosa* in the last year. Mutations predicted to be 'mild' by the biological classification method are associated with more normal sweat chloride levels ($p < 0.001$), pancreatic sufficiency ($p < 0.001$) and decreased risk of

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Conflict of interest

Nothing to declare.

infection with *Pseudomonas* in the last year ($p = 0.014$). Lower Grantham scores are associated with more normal sweat chloride levels ($p < 0.001$), and pancreatic sufficiency ($p = 0.014$). Higher SIFT scores are associated with more normal sweat chloride levels ($p < 0.001$) and pancreatic sufficiency ($p = 0.011$). There was no association between pulmonary function measured by predicted FEV₁ and the biological classification ($p = 0.98$), Grantham ($p = 0.28$) or SIFT scores ($p = 0.62$), which suggests the pulmonary disease related to CF may involve other modifier genes and environmental factors.

Keywords

cystic fibrosis; Grantham; pulmonary function; SIFT; sweat chloride

Cystic fibrosis (CF) is a Mendelian autosomal recessive disease that shows phenotypic heterogeneity and can manifest as chronic sinopulmonary disease, pancreatic insufficiency, elevated sodium chloride loss in sweat, male infertility and gastrointestinal abnormalities, including liver disease (Fig. 1). Pulmonary disease is the major cause of mortality in CF subjects (1) and most clinically relevant phenotype. Over 1500 unique mutations have been identified in the *CFTR* gene (2). The most common allele, p.F508del, has a frequency of over 66% in Caucasians (3). Other mutations have a frequency of less than 5%, although some can be more prevalent in some subpopulations because of founder effects. Because of the vast number of mutations, genetic testing is limited for definitive diagnosis, and the sweat chloride test remains pivotal for diagnosis. Usually, sweat chloride levels above 60 mmol/l are diagnostic (4). There have been multiple efforts to correlate *CFTR* mutation with phenotype to understand the specific effect of each mutation. The biological classification score is based on *in vitro* mutation analysis and is the most accepted classification scheme. Mutations are classified based on known molecular consequences: class I – no synthesis; class II – processing blocked; class III – regulation blocked; class IV – altered conductance; and class V – reduced synthesis (5). Mutations in a higher class are thought to be milder and some class IV and class V mutations have been associated with pancreatic sufficiency (6). Data support a probable correlation between the *CFTR* mutation and *Pseudomonas* infection (7). Missense and splice site mutations that are considered mild CF allelic variants have also been associated with lower risk for acquisition of *Pseudomonas* (8).

We analyzed whether genotype–phenotype correlations exist between *CFTR* mutations and four clinical phenotypes – sweat chloride levels, pancreatic sufficiency, pulmonary function measured by the predicted forced expiratory volume in 1 s (predicted FEV₁), and probability of infection with *Pseudomonas* in the last year. We consider three mutation scoring algorithms – the biological classification, Grantham and Sorting Intolerant from Tolerant (SIFT) scores and assess whether these scores correlate with the disease phenotypes.

Materials and methods

Subjects

We reviewed clinical data from 435 CF subjects followed at Children's Hospital, Boston (CHB). Inclusion criteria required subjects to have two copies of a known *CFTR* mutation or

elevated sweat chloride levels (>60 mmol/l). FEV₁ was measured by standard spirometry according to American Thoracic Society criteria, and absolute values were converted to a percent-age of the predicted volume (predicted FEV₁) expected for a healthy individual of the same age, gender and height (9, 10). Patients were considered pancreatic sufficient according to fecal pancreatic elastase level or evidence of malabsorption with a 24-h fecal fat measurement. The study was approved by the Institutional Review Board at CHB.

Genotyping methods

Genomic DNA was evaluated for the presence of *CFTR* mutations (Genzyme, San Francisco, CA or Ambry Genetics, Aliso Viejo, CA) as part of the clinical evaluation. The entire gene was sequenced. None of the subjects had more than one mutation within each *CFTR* gene and no *cis* mutations were identified.

Statistical methods

The non-p.F508del alleles of the p.F508del compound heterozygotes (one allele is p.F508del and the other is a known non-p.F508del *CFTR* mutation) were classified as ‘mild’ if the biological classification score was class IV or V, or ‘severe’ if the biological classification score was class I, II or III. From henceforth, we refer to this modified biological classification score as the biological classification score.

Next, the analysis was restricted to the non-synonymous missense amino acid mutations. Grantham scores were then calculated for each mutation. Grantham scores are a measure of the chemical dissimilarity between the native and the substituted amino acid and are based on the amino acid substitution matrix of Grantham (11). Higher Grantham scores are usually predictive of severe mutations as defined by *in vitro* assays (12). The SIFT score is correlated with whether the mutation is at an evolutionarily conserved amino acid residue. Mutations that occur in evolutionarily conserved regions are hypothesized to be deleterious and result in a more severe clinical phenotype (13). SIFT score values greater than or equal to 0.05 are predicted as tolerant (14). All non-synonymous missense mutations in the study occurred at evolutionarily conserved sites across the following eight vertebrate orthologs: baboon, macaque, cow, sheep, rabbit, mouse, killifish, and salmon.

Multivariate linear regression models adjusting for age and sex using the biological classification, Grantham and SIFT scores as covariates were used to predict sweat chloride levels and predicted FEV₁. Multivariate logistic regression models using these covariates were used to determine the probability of pancreatic insufficiency and risk of *Pseudomonas* infection in the past year. To ensure robust results, we randomly permuted the biological, Grantham, and SIFT scores 100,000 times to make 100,000 datasets and a permutation test p-value was calculated.

Results

Subjects and genotypes

The genotype distribution for the 435 subjects evaluated is shown in Table 1. A total 24.8% (146) were heterozygous for the p.F508del mutation. Of the 146 p.F508del heterozygotes, a

known *CFTR* mutation was present in 74.0% (108) of the subjects, and the analysis was restricted to these 108 subjects. Twenty-five non-p.F508del alleles were identified (Tables 2 and 3).

Relationship between phenotypes

Predicted FEV₁ has an approximately normal distribution (Shapiro–Wilks $p = 0.61$). Although the predicted FEV₁ data were normalized for age, duration of disease (age) was strongly associated with worse predicted FEV₁ ($p = 1.17 \times 10^{-8}$) suggesting older individuals tend to have worse lung function. Males had higher average predicted FEV₁ and lower average sweat chloride values than females, however, these differences were not statistically significant ($p = 0.14$ and $p = 0.33$, respectively). Males were at lower risk of infection over the past year relative to females (OR = 0.44, $p = 0.044$). Older individuals tended to have higher sweat chloride values ($r = 0.41$, $p = 1.8 \times 10^{-5}$) and higher odds of *Pseudomonas* infection ($p = 7.6 \times 10^{-6}$). No significant correlation existed between predicted FEV₁ and sweat chloride levels ($p = 0.81$) or between sweat chloride concentrations and infection status with *Pseudomonas* after adjusting for age and gender ($p = 0.29$). These results suggest that the sweat chloride level at diagnosis is not a useful marker of lung function. *Pseudomonas* infection within the past year was associated with lower predicted FEV₁ ($p = 1 \times 10^{-4}$) suggesting that lung infections portend worse lung function.

Biological classification score as a predictor of CF phenotypes

Subjects with ‘mild’ mutations tend to have lower sweat chloride levels than subjects with more ‘severe’ mutations ($p < 0.001$). This association persisted after adjusting for age and sex ($p < 0.001$), demonstrating that the *in vitro* *CFTR* function correlates with the *in vivo* sweat chloride levels. There was no significant association between mutation biological classification and predicted FEV₁ on either univariate analysis ($p = 0.37$) or multivariate analysis ($p = 0.98$) (Table 4). Seven of the eleven subjects with ‘mild’ CF genotypes were pancreatic sufficient compared with 5 of 91 subjects with ‘severe’ CF genotypes ($p < 0.001$). This association remained statistically significant after adjusting for age and sex ($p < 0.001$). Subjects with ‘mild’ mutations had a lower probability of *Pseu-domonas* infection in the previous year. Only 3 of the 11 subjects with ‘mild’ CF genotypes had a *Pseu-domonas* infection in the previous year compared to 60 out of 90 subjects with ‘severe’ *CFTR* mutations ($p < 0.001$). This association remained significant after adjusting for age and sex ($p = 0.014$).

Grantham and SIFT scores as predictors of CF phenotypes in p.F508del compound heterozygotes with missense mutations

Higher Grantham scores were associated with higher sweat chloride levels ($p < 0.001$) and positively correlated with pancreatic insufficiency in the univariate ($p = 0.012$) and multivariate analyses ($p = 0.014$). Although there appeared to be an association between higher Grantham scores and increased risk of *Pseu-domonas* infection in the past year in univariate analysis ($p = 0.027$), this association disappeared in the multivariate analysis ($p =$

0.082). There was no significant association between Grantham score and predicted FEV₁ on either univariate ($p = 0.162$) or multivariate analyses ($p = 0.278$).

Univariate analysis showed that higher SIFT scores were associated with lower sweat chloride levels in both univariate ($p < 0.001$) and age and sex adjusted analyses ($p < 0.001$). There was a positive correlation between higher SIFT scores and pancreatic sufficiency in both univariate ($p = 0.009$) and age and sex adjusted analyses ($p = 0.011$). Subjects with higher SIFT scores had a decreased risk of *Pseudomonas* infection in the previous year in the univariate analysis ($p = 0.046$), however, this association was marginal after adjusting for age and sex ($p = 0.058$).

Discussion

On the basis of biological classification score, more 'severe' mutations are associated with higher sweat chloride levels and pancreatic insufficiency. Higher Grantham scores and lower SIFT scores were associated with pancreatic insufficiency and higher sweat chloride levels amongst non-synonymous mutations. The risk of *Pseudomonas* infection in the last year may be associated with the *CFTR* mutation, with the risk increased for subjects with more 'severe' mutations, higher Grantham scores and lower SIFT scores. The genetic scoring methods considered were not significant predictors of predicted FEV₁.

Our findings recapitulate previously published studies that discover no correlation between the pulmonary function of CF subjects measured by predicted FEV₁ and the *CFTR* mutation, and no correlation between the sweat chloride level at age of diagnosis and the predicted FEV₁ (15–19). The data support the *in vitro* biological classification method as a means of classifying the *CFTR* mutations because milder mutations have been associated with *in vivo* pancreatic sufficiency and a milder clinical phenotype (6, 20, 21). Urban et al. found that higher Grantham scores and lower SIFT scores were associated with worse *in vitro* function (12). An analysis of SIFT, Panther and PolyPhen by Dorfman et al. (22) showed that SIFT and Panther are able to predict disease causing mutations, albeit without 100% sensitivity and specificity. Dorfman et al. also showed a positive association between disease severity and SIFT scores, although the association was not significant. These findings suggest that *in silico* tools capture only some of the information in a mutation and should not be used as clinical diagnostic tools. Finally, Kubesch et al. asserted that *CFTR* genotype predicts the risk of airway colonization with *Pseudomonas* (8), possibly directly through its role as an epithelial receptor for this microorganism (7). Murine models of CF have shown these mice have increased susceptibility to colonization with *Pseudomonas* (23). We find borderline evidence to support this hypothesis.

The major clinical implication of our findings is that sweat chloride levels, pancreatic function, and *Pseudomonas* infection risk appear to be associated with the *CFTR* mutation. This suggests treatments that affect the amount of functional CFTR are probably more likely to affect these phenotypes than the pulmonary phenotype. The pulmonary phenotypic heterogeneity in CF subjects with the same genotype is probably due to modifier genes, non-genetic factors or modifier genes interacting with non-genetic factors.

There are a few study limitations. The cohort is a clinical cohort and not a prospective cohort. Data was collected retrospectively and all data collection was performed blinded to the subjects' genotypes, and therefore, not likely to affect the results. The power of the study is limited because of the small sample size and the small numbers of subjects with a given *CFTR* mutation, although our results are very much in concert with previously published reports. The *in vivo* function of the membrane transporter may differ for a different substrate or the membrane transporter may have a non-transport function (12), therefore the biological classification of a mutation may not capture the complete *in vivo* impact of the mutation. In addition to the transport of diverse substrates (18), *CFTR* may have non-transport functions as a receptor for *Pseudomonas* (16). The p.R117H mutation has a variable penetrance as its splicing efficiency is affected by the length of the poly-T-tract in intron 8 (IVS8-5T, 7T and 9T), therefore the genotyping scoring tools are probably inadequate for evaluation of this mutation (24–26). Finally, although we found no association between *CFTR* mutation and predicted FEV₁, further research should be performed following pulmonary status over time, as a longitudinal assessment of pulmonary function may prove to be more informative.

The diversity of lung disease in CF subjects may not be related to variation in *CFTR* mutations, as there is considerable phenotypic heterogeneity, even in subjects with the same genotype or class of *CFTR* mutation. Understanding the complex interplay between mutational variants and phenotype using CF and other monogenic diseases will prove to be important as mutational variants for complex diseases are identified.

Acknowledgments

We are grateful to Dr Sudhir Kumar, who provided us with the data on the evolutionarily conserved domains in the *CFTR* gene. We are also grateful to Solandra Craig, Christopher Garcia and the anonymous reviewers for the critical review of the manuscript. R. S. was supported by a Howard Hughes Medical Institute Pre-Doctoral Fellowship.

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Fig. 1.
Factors that may affect the correlation between CFTR genotype and phenotypes.

Table 1

Genetic distribution of cystic fibrosis (CF) cohort

Number of F508del alleles		<i>n</i> (%)
2	F508del homozygotes	174 (40)
1	One F508del allele and one known non-F508del CFTR mutation ^a	108 (24.8) ^a
	One F508del allele and an unknown mutation	38 (8.7)
0	Two known non-F508del alleles	25 (5.7)
	One known non-F508del allele and one unknown allele	34 (7.8)
	Two unknown alleles	56 (12.9)
Total		435

^aThe subgroup used for the statistical analysis.

Table 2CFTR mutation classification for compound heterozygotes^a

Mutations	n (%)	Biological classification	Grantham score	SIFT
Q493X	3 (3)	I ^b	—	—
G542X	21 (20)	I ^{b, c, e}	—	—
R553X	4 (4)	I ^{b, e}	—	—
Y1092X	2 (2)	I ^b	—	—
R1158X	1 (1)	NA	—	—
W1282X	9 (9)	I ^{b, e}	—	—
G85E	4 (4)	III ^b	98	0.01
R117H	4 (4)	IV ^{b, c}	29	0.60
R334W	1 (1)	IV ^b	101	0.02
R347P	1 (1)	IV ^b	103	0.05
R352Q	1 (1)	NA	43	0.35
G551D	20 (19)	III ^{b, c}	94	0.00
R560T	3 (3)	III ^b	71	0.00
D1270N	1 (1)	NA	23	0.01
N1303K	6 (6)	II ^g	94	0.00
I507del	3 (3)	II ^d	—	—
394delTT	1 (1)	NA ^c	—	—
621+1G>T	7 (7)	I ^{b, f}	—	—
711+1G>T	2 (2)	I ^b	—	—
1717-1G>A	5 (5)	I ^{b, c, e, f}	—	—
1898+1G>A	2 (2)	NA	—	—
2789+5G>A	3 (3)	V ^b	—	—
3659delC	1 (1)	I ^b	—	—
3849+10kbC>T	2 (2)	V ^{b, c, f}	—	—
3905insT	1 (1)	I ^b	—	—

NA, not applicable; SIFT, Sorting Intolerant from Tolerant.

^aThe following mutations biological classification scores could not be verified: 1898+G-A, 394delTT, D1270N, R352Q, and R1158X.^bAhmed et al. (27).^cWilschanski et al. (28).^dWelsh and Smith 29).^eMcKone et al. (21).^fLoubieres et al. (30).^gMoskowiz et al. (31).

Table 3

Biological classification scores for the CFTR mutations in cohort

Mutation classification	Age		Sex		Sweat chloride level		Predicted FEV ₁		Number pancreatic Sufficient		Number infected with <i>Pseudomonas aeruginosa</i> in past year	
	Mean (standard deviation)	Male (%)	Mean (standard deviation)	Female (%)	Mean (standard deviation)	Standard deviation	Mean (standard deviation)	Standard deviation	n (%)	n (%)	n (%)	n (%)
Class I: no synthesis	15.7 (11.5)	25 (45.5)	103.4 (16.2)	86.7 (19.9)	2 (4)		86.7 (19.9)		2 (4)		34 (62)	
Class II: block in processing	17.9 (12.5)	5 (55.6)	109.6 (13.0)	81.1 (25.7)	0 (0.00)		81.1 (25.7)		0 (0.00)		6 (67)	
Class III: block in regulation	16.7 (9.6)	10 (38.5)	107.8 (16.1)	82.1 (17.8)	3 (11)		82.1 (17.8)		3 (11)		20 (77)	
Class IV: altered conductance	17.0 (22.4)	3 (50.0)	69.4 (38.6)	58.5 (9.6)	5 (83)		58.5 (9.6)		5 (83)		1 (17)	
Class V: reduced synthesis	14.6 (14.4)	2 (40.0)	76.4 (23.9)	86.5 (17.1)	2 (40)		86.5 (17.1)		2 (40)		2 (40)	

Table 4Evaluation of *CFTR* mutation scoring methods to predict various components of the CF phenotype

Mutation classification	Analysis	Sweat chloride level (p-value)	Predicted FEV ₁ (p-value)	Pancreatic sufficiency (p-value)	Infected with <i>P. aeruginosa</i> in past year (p-value)
Biological classification score (mild vs severe)	Unadjusted	4.0×10^{-7}	0.371	4.1×10^{-7b}	2.5×10^{-4b}
	Sex and age adjusted	1.4×10^{-8}	0.979	1.4×10^{-5c}	0.014 ^c
Grunham score	Unadjusted	4.8×10^{-5a}	0.162 ^a	0.012 ^c	0.027 ^c
	Sex and age adjusted	3.4×10^{-4a}	0.278 ^a	0.014 ^c	0.082 ^c
SIFT score	Unadjusted	2.1×10^{-7a}	0.330 ^a	0.009 ^c	0.046 ^c
	Sex and age adjusted	<0.001 ^a	0.624 ^a	0.011 ^c	0.058 ^c

CF, cystic fibrosis; SIFT, Sorting Intolerant from Tolerant.

^a p-Values calculated from 100,000 permutations of mutation scores.^b p-Values calculated using Fisher's exact test.^c p-Values obtained from logistic regression.