Cystic fibrosis transmembrane conductance regulator gene mutations and glutathione S-transferase null genotypes in cystic fibrosis patients in Brazil*, **

Mutações do gene *cystic fibrosis transmembrane conductance regulator* e deleções dos genes glutationa S-transferase em pacientes com fibrose cística no Brasil

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Abstract

Objective: To determine the effects that mutation of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene and deletion of the glutathione S-transferase (GST) genes *mu-1* (*GSTM1*) and *theta-1* (*GSTT1*) have on the clinical course of cystic fibrosis (CF) in patients residing in the southeastern region of Brazil. **Methods:** The study sample consisted of all consecutive CF patients treated at the *Hospital de Clínicas* School of Medical Sciences of the State University at Campinas between March of 2002 and March of 2005. We included 66 CF patients. Genomic DNA was analyzed by polymerase chain reaction and restriction endonuclease digestion for the identification of the genotypes. **Results:** The ΔF508 mutation of the *CFTR* gene was found in 44 patients (66.7%). The null genotypes *GSTM1, GSTT1* and *GSTM1/GSTT1* were found in 40.9%, 15.2%, and 3.0% of the patients, respectively. The ΔF508 *CFTR* mutation was more common in patients diagnosed with CF before 2.5 years of age than in those diagnosed later (75.5% vs. 41.2%; p = 0.008). The frequency of the ΔF508 *CFTR* mutation, as well as of the *GSTM1* and *GSTT1* genotypes, was not found to be associated with gender, ethnicity, pulmonary disease status, or pancreatic disease status. **Conclusions:** When the patients were stratified by clinical and epidemiological features, the frequencies of the *GSTM1* and *GSTT1* null genotypes were similar, suggesting that the inherited absence of these enzymatic pathways does not alter the course of CF. However, the high frequency of the ΔF508 *CFTR* mutation found in younger children suggests that it influences the age at diagnosis of CF in this region of Brazil.

Keywords: Cystic fibrosis; Cystic fibrosis transmembrane conductance regulator; Glutathione transferase.

Resumo

Objetivo: Determinar os efeitos que a mutação do gene *cystic fibrosis transmembrane conductance regulator* (*CFTR*) e da deleção dos genes glutationa S-transferase (GST) *mu-1* (*GSTM1*) e *teta-1* (*GSTT1*) têm na evolução clínica da fibrose cística (FC) em pacientes da região sudeste do Brasil. **Métodos:** Entre março de 2002 e março de 2005, incluímos no estudo todos os pacientes consecutivos de FC atendidos no Departamento de Pediatria do Hospital de Clínicas da Faculdade de Ciências Médicas da Universidade Estadual de Campinas. O DNA genômico de 66 pacientes com FC foi analisado por reação em cadeia da polimerase e digestão com endonuclease de restrição para a identificação dos genótipos. **Resultados:** A mutação ΔF508 do gene *CFTR* foi identificada em 44 (66.7%) pacientes. As deleções dos genes *GSTM1*, *GSTT1* e da combinação nula *GSTM1*/*GSTT1* foram identificadas em 40,9%, 15,2% e 3,0% dos pacientes, respectivamente. A mutação ΔF508 do gene *CFTR* foi mais comum em pacientes diagnosticados com FC antes dos 2,5 anos de idade que naqueles diagnosticados mais tarde (75,5% vs. 41,2%; p = 0,008). Foram observadas frequências similares da mutação ΔF508 do gene *CFTR* e dos genótipos *GSTM1* e *GSTT1* nos pacientes, independentemente do sexo, etnia ou status da doença pulmonar ou pancreática. **Conclusões:** Quando os pacientes foram estratificados por aspectos clínicos e epidemiológicos, as frequências dos genótipos *GSTM1* e *GSTT1* nulos foram semelhantes, sugerindo que a ausência herdada dessas vias enzimáticas não altera o curso da FC. Em contraste, a alta frequência da mutação ΔF508 no gene *CFTR* encontrada em pacientes mais jovens sugere que essa mutação influencia a idade no momento do diagnóstico de FC nessa região do país.

Descritores: Fibrose cística; Regulador de condutância transmembrana em fibrose cística; Glutationa transferase.

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Introduction

Cystic fibrosis (CF) is one of the most common severe autosomal recessive genetic diseases among populations of northern European descent. The major clinical manifestations of the disorder are chronic sinopulmonary disease and pancreatic exocrine insufficiency.[1]

There is considerable heterogeneity among individuals with CF in terms of the severity of the disease. Attempts to link phenotypes to specific CF transmembrane conductance regulator (*CFTR*) gene mutations have been successful, particularly for pancreatic disease status.[2-5] This correlation, however, has not completely held for pulmonary disease, which can vary markedly among patients with the same *CFTR* gene mutation.[6] The severity of pulmonary disease in CF seems to be influenced by other mutations located out of the *CFTR* gene locus,[7,8] such as the those occurring in the glutathione S-transferase (GST) enzymes.[9-13] Glutathione is a major local pulmonary antioxidant that is present in the epithelial lining fluid. The GST enzymes detoxify harmful organic hydroperoxides that are formed as a result of exposure to oxidant stress, such as those found in the lungs of patients with CF,[14] by conjugating them with glutathione, thereby potentially preventing further pulmonary damage.

The genes *GST mu-1* (*GSTM1*) and *GST theta-1* (*GSTT1*) are polymorphic in humans and are absent or homozygous null, resulting in a lack of active proteins, in approximately 40% and 20% of normal individuals, respectively.[15] In CF, there is no consensus regarding the roles of the *GSTM1* and *GSTT1* null genotypes in the severity of lung disease[9-13] and pancreatic disease.[9]

Among Whites in Brazil, CF is one of the most common severe inherited diseases.[16] Short survival due to pulmonary disease has been described in CF patients treated at our hospital.[16] However, the modifiers of disease severity, if any, are still unknown. Therefore, we considered it necessary to identify the *CFTR* mutation, as well as the *GSTM1* and *GSTT1* genotypes, in CF patients in Brazil, in order to determine whether these genetic factors influence the clinical course of the disease in the southeastern region of the country.

Methods

The study sample consisted of all consecutive CF patients treated in the *Hospital de Clinicas* of the State University of Campinas, located in the city of Campinas, Brazil, between March of 2002 and March of 2005. The diagnosis of CF was based on signs and symptoms, such as failure to thrive, meconium ileus, vomiting, abdominal pain, diarrhea, steatorrhea, and recurrent pulmonary infections. Two tests of chloride concentration in sweat, with results equal to or greater than 60 mEq/L and performed by the classical pilocarpine test,[17] were required for confirmation of the diagnosis. We considered the age at diagnosis of CF, as well as the current severity of the pulmonary and pancreatic diseases, as measured by the Shwachman score.[18] All procedures were carried out in accordance with the ethical principles of the institutional guidelines, and all patients or their relatives gave written informed consent.

Genomic DNA of peripheral blood was used for the genotyping of all CF patients. The ΔF508 *CFTR* mutation was detected directly by polymerase chain reaction (PCR; Figure 1a).[19] The G542X, G551D, R553X, R1162X, W1282X, and N1303K mutations of the *CFTR* gene were analyzed by restriction endonuclease digestion of the PCR products.[20] The genes *GSTM1*, *GSTT1* and β-*globin* (as a reaction control) were amplified by multiplex-PCR (Figure 1b).[21]

The patients were stratified by the pattern of *CFTR* gene mutation (homozygous ΔF508 mutation, heterozygous ΔF508 mutation, and other or unknown *CFTR* mutations) and *GSTM1* and *GSTT1* gene mutations (present or with homozygous deletion). We used the chi-square test or Fisher's exact test to calculate the statistical significance of differences in age (< 1.0 year vs. ≥ 1.0 year; and < 2.5 years vs. ≥ 2.5 years); gender (male vs. female); ethnic origin (White vs. African-Brazilian); and lung and pancreatic exocrine status (excellent/good/mild vs. moderate/severe); and *GSTM1* and *GSTT1* genotypes (present vs. null), isolated or in combination. The associations of genotypes and clinical variables were also evaluated by multivariate analysis using a logistic regression model. Factors with a $p \leq 0.05$ were considered statistically significant.

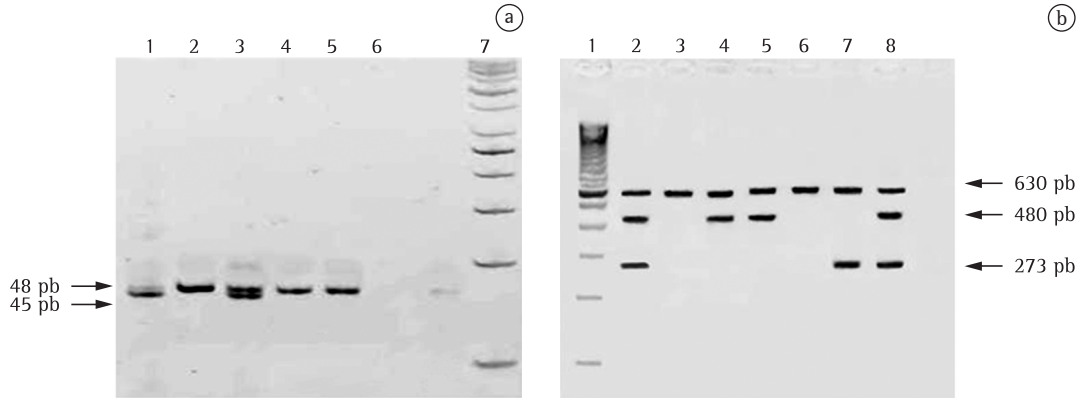

Figure 1 - Polymerase chain reaction for the detection of the ΔF508 mutation in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, as well as of the deletion of glutathione S-transferase (GST) genes *mu-1* (*GSTM1*) and *theta-1* (*GSTT1*), in cystic fibrosis. In a, 12% polyacrylamide gel stained with ethidium bromide showing 45-bp and 48-bp fragments, corresponding to the mutant and normal alleles, respectively. Lane 1 shows the result from an individual with the homozygous ΔF508 mutation; lanes 2, 4, and 5 show the results of individuals without the ΔF508 mutation, whereas lane 3 shows the result from a heterozygous individual. A control sample without DNA and a 10-bp DNA ladder are presented in lanes 6 and 7, respectively. In b, 2% agarose gel stained with ethidium bromide showing 273-bp, 480-bp, and 630-bp fragments, corresponding to the genes *GSTM1*, *GSTT1*, and β-*globin* (as a reaction control), respectively. Lane 1 also shows a 100-bp DNA ladder. Lanes 2 and 8 show the results from individuals with the *GSTM1* and *GSTT1* genes. Lanes 3 and 6 show a combined homozygous *GSTM1* and *GSTT1* deletion. Lanes 4 and 5 show the results from individuals with homozygous deletion of *GSTM1*, whereas lane 7 shows the results from an individual with homozygous deletion of *GSTT1*.

All analyses were performed using the statistical package SAS System for Windows, version 8.1 (SAS Institute, Cary, NC, USA).

Results

The study involved 66 patients with CF (Table 1). The median age of the patients at diagnosis was 1.0 year (range, 0.1-15.0 years). At the time of CF diagnosis, 27 of the patients were younger than 1.0 year of age, 39 were at least 1.0 year old, 49 were under 2.5 years of age, and 17 were over 2.5 years of age. Males and females were similarly represented in the study. Almost all individuals were White. Moderate and severe forms of CF were found in 57.6% of the patients. The prevalences of these forms of the disease were similar in patients under and over 1.0 year of age (59.2% and 56.4%, respectively; p = 1.00), as well as in those under and over 2.5 years of age (61.2% and 47.1%, respectively; p = 0.40).

The ΔF508 *CFTR* mutation was identified in 44 of the 66 patients (66.7%). The homozygous and heterozygous mutations were seen in 17 (25.8%) and in 27 (40.9%), respectively. The G542X, R1162X, and N1303K mutations of the *CFTR* gene were found in heterozygosis in 8 (12.1%), 2 (3.0%), and 1 (1.5%) of the patients, respectively. The G551D, R553X, and W1282X mutations were not found in any of our patients. We found that 19 of the patients did not have any of the mutations studied. The allelic frequencies of the ΔF508, G542X, R1162X, and N1303K mutations of the *CFTR* gene were 0.462, 0.061, 0.015, and 0.008, respectively. The frequencies of the homozygous and heterozygous ΔF508 *CFTR* mutations were similar in patients under and over 1.0 year of age (34.8% and 31.8%, respectively; p = 0.18). However, such mutations were more common in patients under 2.5 years of age than in the older patients (75.5% vs. 41.2%; p = 0.008). No significant differences regarding gender, ethnic origin, or lung/exocrine pancreatic disease status were observed among the CF patients in relation to the distinct patterns of the *CFTR* mutation. Similar frequencies of the G542X, R1162X, and N1303K mutation patterns of the *CFTR* gene (data not shown), as well as of the *GSTM1*, *GSTT1*, and *GSTM1*/*GSTT1* null genotypes, were seen in patients under and over 1.0 year of age (data not shown). The same was

Table 1 - The mutations of the *CFTR* gene and *GSTM1* and *GSTT1* null genotypes in relation to the clinical variables in cystic fibrosis patients.

Variable	n	*CFTR* mutation		*GSTM1*	*GSTT1*	*GSTM1*/*GSTT1*	
		With ΔF508	Without ΔF508	Null	Null	One null	Both null
		n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Age, years[a]							
< 2.5*	49	37 (75.5)	12 (24.5)	22 (44.9)	6 (12.2)	26 (53.1)	1 (2.0)
≥ 2.5*	17	7 (41.2)	10 (58.8)	5 (29.4)	4 (23.5)	7 (41.2)	1 (5.9)
Gender	66						
Male	35	22 (62.9)	13 (37.1)	16 (45.7)	7 (20.0)	19 (54.3)	2 (5.7)
Female	31	22 (71.0)	9 (29.0)	11 (35.4)	3 (9.7)	14 (45.2)	0 (0.0)
Ethnic group	66						
White	65	43 (66.2)	22 (33.8)	27 (41.5)	9 (13.9)	32 (49.2)	2 (3.1)
African-Brazilian	1	1 (100.0)	0 (0.0)	0 (0.0)	1 (100.0)	1 (100.0)	0 (0.0)
Lung disease status[b]	66						
Mild[c]	36	24 (66.7)	12 (33.3)	16 (44.4)	6 (16.7)	20 (55.6)	1 (2.8)
Severe[d]	30	20 (66.7)	10 (33.3)	11 (36.7)	4 (13.3)	13 (43.3)	1 (3.3)
Exocrine pancreatic disease status[b]	66						
Mild[c]	28	18 (64.3)	10 (35.7)	12 (42.9)	5 (17.9)	15 (53.6)	1 (3.6)
Severe[d]	38	26 (68.4)	12 (31.6)	15 (39.5)	5 (13.2)	18 (47.4)	1 (2.6)

[a]Age at diagnosis of the disease. [b]Defined using the Shwachman score. [c]Including excellent, good, and mild status. [d]Including moderate and severe status. *p = 0.02 for the comparison of frequencies of homozygous and heterozygous ΔF508 *CFTR* mutation in patients under and over 2.5 years of age; OR = 5.0 (95% CI: 1.5-16.7), and p = 0.008 was obtained for the same analysis when results were adjusted by the multivariate analysis.

true for patients under and over 2.5 years of age at diagnosis, regardless of gender, ethnic origin, or lung/exocrine pancreatic disease status (Table 1). Similar frequencies of the distinct patterns of the *CFTR* mutations in combination with the genotypes *GSTM1* null, *GSTT1* null, and *GSTM1*/*GSTT1* null were seen in patients under and over 1.0 year of age (data not shown). After multivariate analysis, the frequencies of the genotypes were also similar among patients stratified by age (< 2.5 and ≥ 2.5 years of age), gender, ethnic origin, and lung/exocrine pancreatic disease status (Table 2). No differences in age, gender, ethnic origin, or lung/exocrine pancreatic disease status were seen among the patients with the G542X, R1162X, or N1303K mutations in the *CFTR* gene combined with the *GSTM1*, *GSTT1*, or *GSTM1*/*GSTT1* null genotypes (data not shown).

Discussion

The distribution of the patients enrolled in the study by age at diagnosis, gender, ethnic origin, and severity of lung/exocrine pancreatic disease status showed that our CF patients were similar to those in other countries.[1] The ΔF508 *CFTR* gene mutation was the most common mutation found in our study, which corroborates other reports from the same region of Brazil,[16,22-25] as well as international reports.[26,27] Other mutations of the *CFTR* gene, such as R1162X and N1303K, were less common in our CF patients,[22,24,28] as has been reported for CF patients in other parts of the world.[27] The frequencies of the *GSTM1* and *GSTT1* null genotypes in the CF patients in our study were similar to those found in healthy individuals, in Brazil[29] and elsewhere.[15] Therefore, our sample seems to be representative of CF patients throughout the world.

We found a high frequency of the ΔF508 *CFTR* mutation in patients diagnosed with the disease before 2.5 years of age in comparison with those diagnosed later. However, we found no difference in the frequencies of the gene mutation in patients stratified by lung/pancreatic disease status. Because the frequencies of the moderate and severe forms of CF were similar in the patients under and over 2.5 years of age, the combination of the ΔF508 *CFTR* gene mutation and the diagnosis of the disease at a younger age suggests that the mutation provokes more symptoms or makes symptoms more persistent,

Table 2 - The ΔF508 *CFTR* gene pattern combined with the *GSTM1* and *GSTT1* null genotypes in cystic fibrosis patients stratified by clinical variables.

Variable	*GSTM1* null		*GSTT1* null		*GSTM1*/*GSTT1* null	
	With ΔF508	Without ΔF508	With ΔF508	Without ΔF508	With ΔF508	Without ΔF508
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Age, years[a]						
< 2.5*	16 (72.7)[d]	6 (27.3)	6 (100.0)	0 (0.0)	1 (100.0)	0 (0.0)
≥ 2.5*	1 (20.0)	4 (80.0)	2 (50.0)	2 (50.0)	0 (0.0)	1 (100.0)
Gender						
Male	9 (56.3)	7 (43.8)	6 (85.7)	1 (14.3)	1 (50.0)	1 (50.0)
Female	8 (72.7)	3 (27.3)	2 (66.7)	1 (33.3)	0 (0.0)	0 (0.0)
Ethnic group						
White	17 (63.0)	10 (37.0)	7 (87.5)	2 (12.5)	1 (50.0)	1 (50.0)
African-Brazilian	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
Lung disease status[b]						
Mild[c]	11 (68.8)	5 (31.3)	6 (100.0)	0 (0.0)	1 (100.0)	0 (0.0)
Severe[d]	6 (54.5)	5 (45.5)	2 (50.0)	2 (50.0)	0 (0.0)	1 (100.0)
Exocrine pancreatic disease status[b]						
Mild[c]	9 (75.0)	3 (25.0)	5 (100.0)	0 (0.0)	1 (100.0)	0 (0.0)
Severe[d]	8 (53.3)	7 (46.7)	3 (60.0)	2 (40.0)	0 (0.0)	1 (100.0)

[a]Age at diagnosis of the disease. [b]Defined using the Shwachman score. [c]Including excellent, good, and mild status. [d]Including moderate and severe status. *p = 0.06 for the comparison of the frequencies of the *GSTM1* null genotype and ΔF508 *CFTR* mutation in patients under and over 2.5 years of age; OR = 14.05 (95% CI: 0.76-260.25), and p = 0.76 was obtained for the same analysis when results were adjusted by the multivariate analysis.

which might prompt the parents of patients with this mutation to seek medical advice sooner.

The ΔF508 mutation results in the production of an abnormally folded *CFTR* that is not trafficked normally to the apical cell membrane.[30] In fact, there is evidence that the gene abnormality alone does not affect the severity of the lung disease.[6,16,26] This seems to be influenced by other mutations located out of the *CFTR* locus. In addition, the association between the ΔF508 mutation and the severity of pancreatic exocrine insufficiency in CF patients has been consistently demonstrated.[2,5] Therefore, it is noteworthy that our sample size (n = 66) might not have been sufficient to detect associations between the ΔF508 mutation in the *CFTR* gene and the clinical features, and that the method used for determining the lung/pancreatic disease status (the Shwachman score) might have been inappropriate for the purposes of this study. In addition, a second mild allele accompanying the ΔF508 mutation might have protected our patients against severe pancreatic disease, as previously described.[2,5] It is also possible that our patients did not survive long enough to present pancreatic dysfunction.

We found no differences between the patients with the *GSTM1* or *GSTT1* genes and those without in terms of age, gender, ethnic origin, or lung/pancreatic disease status, suggesting that the GST enzymes do not alter the clinical characteristics of CF patients in our region. The GST multigene family of detoxifying enzymes is involved in protecting various tissue types from oxidative damage. The homozygous *GSTM1* null genotype has been associated with greater lung disease severity and decreased survival in CF patients,[9-11,13] although there are conflicting data.[12] In addition, we found that the inherited absence of the *GSTM1*[9] and *GSTT1*[11,12] enzymes was not associated with the severity of pancreatic or lung disease. Although the absence of these GST proteins might have altered the clinical function of our CF patients, their influence might not have been strong enough to be detected in our sample. Furthermore, the ΔF508 *CFTR* mutation combined with the *GSTM1* or *GSTT1* null genotypes were also equally distributed among our CF patients, stratified by the clinical

characteristics. However, our sample size was too small to permit consistent conclusions to be drawn regarding the association of these genotypes with the clinical manifestations of the disease.

In conclusion, our data provide preliminary evidence that the GST detoxifying enzymes do not influence the course of CF, but that the ΔF508 mutation alters the age at diagnosis of the disease. However, epidemiological studies involving larger samples of patients should be carried out in order to clearly identify the roles that these mutations play in the population of CF patients in Brazil.

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