Cystic fibrosis transmembrane conductance regulator mutations at a referral center for cystic fibrosis*

Mutações no gene *cystic fibrosis transmembrane conductance regulator* em um centro de referência para a fibrose cística

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Abstract

Objective: To determine the frequency of six mutations (F508del, G542X, G551D, R553X, R1162X, and N1303K) in patients with cystic fibrosis (CF) diagnosed, at a referral center, on the basis of abnormal results in two determinations of sweat sodium and chloride concentrations. **Methods:** This was a cross-sectional study involving 70 patients with CF. The mean age of the patients was 12.38 ± 9.00 years, 51.43% were female, and 94.29% were White. Mutation screening was performed with polymerase chain reaction (for F508del), followed by enzymatic digestion (for other mutations). Clinical analysis was performed on the basis of gender, age, ethnicity, pulmonary/gastrointestinal symptoms, and Shwachman-Kulczycki (SK) score. **Results:** All of the patients showed pulmonary symptoms, and 8 had no gastrointestinal symptoms. On the basis of the SK scores, CF was determined to be mild, moderate, and severe in 22 (42.3%), 17 (32.7%), and 13 (25.0%) of the patients, respectively. There was no association between F508del mutation and disease severity by SK score. Of the 140 alleles analyzed, F508del mutation was identified in 70 (50%). Other mutations (G542X, G551D, R553X, R1162X, and N1303K) were identified in 12 (7.93%) of the alleles studied. In F508del homozygous patients with severe disease, the OR was 0.124 (95% CI: 0.005-0.826). **Conclusions:** In 50% of the alleles studied, the molecular diagnosis of CF was confirmed by identifying a single mutation (F508del). If we consider the analysis of the six most common mutations in the Brazilian population (including F508del), the molecular diagnosis was confirmed in 58.57% of the alleles studied.

Keywords: Cystic fibrosis; Cystic fibrosis transmembrane conductance regulator; Mutation.

Resumo

Objetivo: Determinar a frequência de seis mutações (F508del, G542X, G551D, R553X, R1162X e N1303K) em pacientes com fibrose cística (FC) de um centro de referência, diagnosticados pela presença de duas dosagens de sódio e cloro no suor alteradas. **Métodos:** Estudo de corte transversal com 70 pacientes com idade média de 12,38 ± 9,00 anos, sendo que 51,43% eram do sexo feminino, e 94,29% eram caucasoides. A triagem de mutações foi realizada pela técnica de reação em cadeia da polimerase (F508del), seguida por digestão enzimática (demais mutações). A análise clínica foi realizada utilizando as variáveis sexo, idade, etnia, manifestações pulmonares/digestivas e escore de Shwachman-Kulczycki (ESK). **Resultados:** Todos os pacientes apresentaram manifestações pulmonares, e 8 não apresentaram manifestações digestivas. Os resultados do ESK evidenciaram doença leve, moderada e grave, respectivamente, em 22 (42,3%), 17 (32,7%) e 13 (25,0%) pacientes. Não houve associação da mutação F508del com o grau de doença pelo ESK. Dos 140 alelos analisados, a mutação F508del foi identificada em 70 (50%). As demais mutações (G542X, G551D, R553X, R1162X e N1303K) foram identificadas em 12 (7,93%) dos alelos analisados. Em pacientes homozigotos F508del com doença grave, a OR foi de 0,124 (IC95%: 0,005-0,826). **Conclusões:** O diagnóstico molecular de FC foi confirmado pela identificação de apenas uma mutação (F508del) em 50% dos alelos estudados. Se considerarmos a análise das seis mutações de maior frequência na população brasileira (incluindo F508del), o diagnóstico molecular foi confirmado em 58,57% dos alelos analisados.

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

Introduction

Cystic fibrosis (CF; #219700) is an autosomal recessive monogenic disorder caused by mutations in the cystic fibrosis transmembrane regulator (*CFTR*; #602421) gene in region 7q3.1.[1] Since the *CFTR* gene was identified, approximately 2,000 mutations have been identified in it.

Currently, the diagnosis of CF is based on the finding of abnormal sweat sodium and chloride concentrations; however, other diagnostic tools have been studied, such as: (a) determination of salivary sodium and chloride concentrations[2]; induction of chloride secretion in response to β-adrenergic stimulation of sweat[3]; (c) *CFTR*-mediated chloride secretion in rectal biopsies[4]; (d) neonatal screening by immunoreactive trypsin[5]; (e) measurement of nasal potential difference[6]; and (f) next-generation sequencing of or identification of two mutations in the *CFTR* gene following (or not) neonatal screening.[7-9]

The severity of CF depends on and is modulated by environmental factors, modifier genes, and classes of mutations in the *CFTR* gene.[8-17] Mutations in the *CFTR* gene are divided into six classes associated with disease severity.[8,9] Class I, II, and III mutations result in greater severity than do class IV, V, and VI mutations.

A study of all mutations in the *CFTR* gene is not possible in Brazil and in many countries. In this context, the present study sought to identify the most common mutations in the *CFTR* gene at a referral center. All patients were screened for the most common mutations in CF—F508del (c.1521_1523delCTT; p.Phe508del; class II); G542X (c.1624G>T; p.Gly542X; class I); G551D (c.1652G>A; p.Gly551Asp; class III); R553X (c.1657C>T; p.Arg553X; class I); R1162X (c.3484C>T; p.Arg1162X; class I); and N1303K (c.3909C>T; p.Asn1303Lys; class II)—and the results were compared with patient clinical and demographic characteristics.

Methods

This was a cross-sectional study involving 70 patients with CF diagnosed on the basis of two sweat chloride test results of 60 mEq/L or greater.

The study was approved by the local research ethics committee (Protocol no. 297/2003). All patients, or parents/legal guardians of patients who were minors, gave written informed consent before the beginning of the study.

Extraction of DNA was performed using the lithium chloride technique. The DNA concentration used for the analysis was 50 ng/mL, evaluated in a spectrophotometer (GE NanoVue™; GE Healthcare Biosciences, Pittsburgh, PA, USA).

The procedure used for identification of mutations in the *CFTR* gene, including primers, fragment sizes, restriction enzymes (in accordance with the manufacturer's guidelines), and the fragment that represents the presence of the mutation, is described in Tables 1 and 2.

The technique used for identification of the mutations analyzed (G542X, G551D, R553X, R1162X, and N1303K) was enzymatic digestion, except for mutation F508del, which is the deletion of three base pairs on exon 10. On exon 11 (G542X, G551D, and R553X), when the presence of mutations is detected, a second enzymatic digestion is performed to differentiate mutations G551D and R553X.

The clinical characteristics of the patients were investigated by two of the authors of the study, who were qualified to perform the analysis. The following data were used for the study: gender (male/female); age (< 10 years, 11-20 years, and > 21 years); ethnicity (White and non-White); clinical symptoms of pulmonary involvement (as determined by culture of respiratory tract secretions, spirometry, and transcutaneous oxygen saturation measurements); gastrointestinal symptoms (as determined by fecal fat balance studies and fecal elastase measurements); and Shwachman-Kulczycki (SK) score.

The SK score assesses nutrition, general activity, physical examination, and radiographic changes. For each item evaluated, the maximum score is 25 points. Lower scores translate to poorer clinical status. The total score is graded as very mild (86-100), mild (71-85), moderate (56-70), severe (41-55), and extremely severe (40 or less).[18] In our study, we defined mild disease as an SK score of 71 to 100, moderate disease as an SK score of 56 to 70, and severe disease an SK score of 55 or less. The scores were categorized this way in order to obtain a smaller number of groups to allow the statistical analysis of the data.

The statistical analysis was performed with the IBM SPSS Statistics software package, version 21.0 (IBM Corporation, Armonk, NY, USA).

Mutations, as well as categorical clinical and demographic data (gender, ethnicity, and presence of pulmonary/gastrointestinal symptoms), were expressed as absolute and percentage values. Age was expressed as mean and standard deviation.

In order to compare clinical severity in terms of identification of the *CFTR* gene mutations included in this study, the chi-square test and Fisher's exact test, including calculation of OR, were used for comparisons between presence of mutations and patient clinical and demographic characteristics.

Table 1 – Reagent concentrations and programs used in polymerase chain reaction for identification of mutations in the cystic fibrosis transmembrane regulator gene in patients with cystic fibrosis.

Reagents	Exon 10	Exon 11	Exon 19	Exon 21
	F508del	G542X, G551D, and R553X	R1162X	N1303K
DNA, µL	1.0	1.0	1.0	1.0
Tris-HCl (pH 8.4), 10.0 mM	2.5	2.5	2.5	2.5
KCl, 25.0 mM	2.5	2.5	2.5	2.5
$MgCl_2$, 1.0 mM	2.0	4.0	1.0	2.0
dNTP, 0.025 mM (each)	1.0	1.0	0.8	2.0
Primers, 0.2 pmol (each)	1.0	1.0	0.8	1.0
Taq polymerase, 5 IU	1.0	1.0	1.0	1.0
Water q.s., mL	25.0	50.0	50.0	50.0
Programs, temperature/time				
1	94°C/5'	95°C/1'	94°C/3'	94°C/5'
2	94°C/1'	50°C/1'	94°C/1'	94°C/30''
3	52.5°C/1'	74°C/2'	56°C/1'	53°C/30''
4	72°C/2'	74°C/9'	72°C/2'	72°C/30''
5	72°C/7'	72°C/7'	72°C/7'	72°C/7'
Cycles, n	35	35	35	35

q.s.: quantity sufficient.

Table 2 – Sequence of primers and digestive enzymes used in polymerase chain reaction and in enzymatic digestion for identification of mutations in the cystic fibrosis transmembrane regulator gene in patients with cystic fibrosis.

Exon	Mutation	Sequence 5' - 3'	Fragment, pb	First digestion				Second digestion		
				Restriction enzyme	Temperature, °C	Normal fragment, pb	Mutant fragment, pb	Restriction enzyme	Temperature, °C	Mutant fragment, pb
11	G542X	S: CAGAGAAAGACAATATAGTTCC	112	*Bstn*I	60	90, 22	112			
	G551D R553X	AS: AAATGCTTGCTAGACCAAT		*Hind*I	37	58, 54	112	*Mbo*I	37	62, 50 / 112
19	R1162X	S: GCCCGACAAATAACCAAGTGA	454	*Dde*I	37	275, 179	179, 143, 132			
		AS: GCTAACACATTGCTTCAGGCT								
21	N1303K	S: CCACTGTTCATAGGGATCCAG	59	*Bstn*I	60	40, 19	59			
		AS: AGAAAGTATTTATTTTTTCTGGAAC								
10	F508del	S: GGC ACC ATT AAA GAA AAT ATC	50, 47							
		AS: CTA TAT TCA TCA TAG GAA AC									

Table 3 – Association between the genotype of the cystic fibrosis transmembrane regulator gene for mutation F508del and gastrointestinal symptoms.

Genotype for the F508del mutation	Gastrointestinal clinical signs			OR	95% CI
	Yes[a]	No[a]	Total, n		
F508del/F508del	21 (33.87)	0 (-)	21	-	-
F508del/another mutation	26 (41.94)	2 (25)	28	2.145	0.417-16.50
Another mutation/another mutation	15 (24.19)	6 (75)	21	0.111	0.014-0.581

[a]Values expressed as n (%).

Table 4 – Description of the mutations in the cystic fibrosis transmembrane regulator gene identified in the patients with cystic fibrosis.[a]

Genotype	Patients
F508del/F508del	21 (30.00)
F508del/another mutation	20 (28.57)
F508del/G542X	3 (4.29)
G542X/R1162X	1 (1.43)
G542X/another mutation	2 (2.86)
F508del/R553X	1 (1.43)
F508del/R1162X	2 (2.86)
F508del/N1303K	2 (2.86)
Another mutation/another mutation	18 (25.71)
Allele	Frequency
F508del	70 (50.00)
G542X	6 (4.29)
R1162X	3 (2.14)
N1303K	2 (1.43)
R553X	1 (0.07)
Not determined	58 (41.43)

[a]Values expressed as n (%).

Results

The mean age of the participants was 12.38 ± 9.00 years (range, 2-49 years). Patient distribution by age group was as follows: < 10 years, 57.14%; 11-20 years, 27.14%; and > 21 years, 15.72%.

There were 36 female patients (51.43%). The distribution by gender was uniform.

Most patients were of White origin (94.29%). All patients had respiratory symptoms, and only 8 (11.43%), of whom 2 were compound heterozygous for mutation F508del and 6 did not have this mutation (OR = 0.111; 95% CI: 0.014-0.581), had no gastrointestinal symptoms (Table 3).

The genotypes and allele frequency for the mutations identified are described in Table 4. In the 52 patients with genotypes containing at least one of the mutations studied, 22 (42.3%), 17 (32.7%), and 13 (25.0%), respectively, were classified as having mild, moderate, and severe disease on the basis of the SK scores. There was no association between mutation F508del and disease severity as measured by SK score; only F508del homozygous patients showed an OR of 0.124 (95% CI: 0.005-0.826) for the severe form (Table 5).

Mutation F508del was identified in 70 (50%) of the 140 alleles analyzed. The genotype frequency analysis showed that homozygosity for mutation F508del occurred in 30% of the patients.

Discussion

The values of the age distribution of the patients in our study were similar to those of Maróstica et al.[19] In 61 patients, ages ranged from 4 months to 17 years, and 73.77% of those patients were under 10 years of age. This distribution should reflect the presence of class I-III mutations, which are more frequently identified in pediatric CF centers than in adult

Table 5 – Association between the genotype of the cystic fibrosis transmembrane regulator gene for the F508del mutation and disease severity by Shwachman-Kulczycki score.

Mutation	Disease severity						Total
	Severe[a]	OR (95% CI)	Moderate[a]	OR (95% CI)	Mild[a]	OR (95% CI)	
F508del/F508del	1 (7.69)	0.124 (0.005-0.826)	9 (40.91)	1.88 (0.57-6.33)	7 (41.18)	1.731 (0.495-5.995)	17
F508del/another mutation	8 (61.54)	2.512 (0.684-9.926)	9 (40.91)	0.795 (0.253-2.453)	6 (35.29)	0.584 (0.166-1.939)	23
Another mutation/another mutation	4 (30.77)	1.703 (0.371-7.128)	4 (18.18)	0.617 (0.141-2.393)	4 (23.53)	1.038 (0.234-4.124)	12

[a]Values expressed as n (%).

CF centers, where there is a higher prevalence of class IV-VI mutations.

Since CF is an autosomal recessive genetic disease, it is expected that there be a balance in prevalence between genders, as was observed by us. Some authors have described a slight male predominance, a finding reported by Streit et al.[20] and Maróstica et al.,[19] respectively, with values of 61% and 62.3% for the male gender. One hypothesis is the greater deterioration in lung function observed in girls during puberty.

The prevalence of Whites is in agreement with the literature, which shows a low incidence of CF among non-Whites. In Brazil, studies conducted in the state of Rio Grande do Sul (predominantly White)[19] and in the state of Bahia (predominantly Black)[21] reported 100% and 28.7% of Whites with CF, respectively, showing that the prevalence of CF might be associated with ethnic characteristics.

Although the molecular diagnosis of CF is complex because of the molecular heterogeneity of the *CFTR* gene, a point analysis of some mutations is necessary. A study involving screening for mutation F508del as a first step for molecular identification of mutations in the *CFTR* gene was performed by our group.[7] Mutation F508del was identified in 70 (50%) of the 140 alleles analyzed. The high prevalence of mutation F508del can also be observed in studies conducted in the Brazilian states of Rio Grande do Sul,[20] São Paulo,[22,23] Rio de Janeiro,[24] and Pará,[25] in which, respectively, the analysis of 154, 116, 148, 108, and 66 alleles showed a frequency of 48.7% (χ^2 = 0.05; p = 0.82), 47% (χ^2 = 0.17; p = 0.68), 44.45% (χ^2 = 0.75; p = 0.38), 25.68% (χ^2 = 18.16; p < 0.01), and 22.7% (χ^2 = 13.77; p = 0.01). It is of note that all of those studies were conducted in Brazil.

The higher prevalence rates of homozygosity and heterozygosity for mutation F508del as compared with those observed in other Brazilian states underscores the importance of screening for mutation F508del in Brazil.[7] Whereas in our study the genotype frequency analysis showed that homozygosity for mutation F508del occurred in 30% of the patients, in the studies conducted in Rio Grande do Sul,[20] São Paulo,[23] and Rio de Janeiro,[24] homozygosity for this mutation occurred in 31.2%, 21.3%, and 10.81% of the patients, respectively. In contrast, in our study, heterozygosity occurred in 57.14%, which is a higher value than those found in the same aforementioned studies (28.6%, 46.3%, and 22.97%, respectively).[20,23,24]

The prevalence of mutation G542X was 4.29% in our study, a value that is similar to those found in other studies conducted in Brazil—3.2% (χ^2 = 6.22; p = 0.63),[20] 2.7% (χ^2 = 0.54; p = 0.46),[24] and 5.5 (χ^2 = 11.91; p < 0.01).[26] Mutation R553X was identified in 0.71% of the alleles analyzed. In the studies by Streit et al.[20] and Raskin et al.,[26] the frequency of mutation R553X was 0.7% (χ^2 = 0.01; p = 0.93) and 0.8% (χ^2 = 4.31; p = 0.037), respectively. Mutation R1162X was detected in 2.4% of the alleles. Although class I mutations (G542X, R553X, and R1162X) are of low prevalence in Brazil (being identified in less than 6% of the alleles studied), it is of note that studies in animal models and humans have been promising for new drugs to correct CFTR function for these mutations, among which is PTC124.[27]

Unlike mutation F508del, which is also a class II mutation and was highly prevalent, mutation N1303K was of low prevalence (being identified in 1.43% of the alleles analyzed). Other studies conducted in Brazil did not analyze this mutation.[20,26] For this class of mutation, drugs to correct and potentiate CFTR function, such as VX-770 and VX-809, have been studied.[28,29]

Mutation G551D was not detected in the sample evaluated, being only identified by Raskin et al.[26] in one allele analyzed (1.8%; χ^2 = 2.51; p = 0.11). For this mutation, VX-770, a drug known as kalydeco (Vertex®), is available on the market.[28]

In our sample of 70 patients with CF, 8 patients had no gastrointestinal symptoms. Of those, none were homozygous for F508del, and, in 5, none of the mutations studied were identified.

Although it is known that there is a relationship between mutation F508del and disease severity, in the association of this mutation with the SK scores, 1.92% of the individuals who were homozygous for mutation F508del had severe disease, whereas 23.08% of the individuals with two undiagnosed mutations had severe disease as measured by SK score. Some hypotheses can be formulated to explain the lower frequency of severe disease as measured by SK score in F508del homozygous patients, such as gene modulation,[8-17] a higher early mortality rate in these patients, and low adherence to disease management or treatment.

In conclusion, the analysis of the six mutations in the *CFTR* gene enabled the confirmation of the molecular diagnosis of CF in 58.57% of the 140 alleles analyzed. However, 50% of the total number of alleles analyzed carried mutation F508del, and screening for this mutation should be the first step for identification of mutations in the *CFTR* gene, on the basis of the recent implementation of the neonatal screening test in our state. The presence of SK scores indicating mild disease in most patients, even in those who were homozygous for class II mutations, could be due to the low age of the patients, who still do not exhibit the clinical severity associated with disease progression, as well as to low adherence to treatment among the patients with greater disease severity.

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