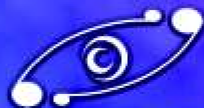

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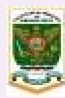

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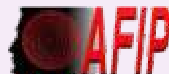

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Frequency of 8 CFTR gene mutations in cystic fibrosis patients in Minas Gerais, Brazil, diagnosed by neonatal screening

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Abstract

The nature and frequency of cystic fibrosis mutations in Brazil is not uniform due to the highly varied ethnic composition of the population. The average frequency of the F508del mutation has been reported to be 48.6%. Other common mutations in Brazil are G542X, R1162X, and N1303K. The aim of this study was to analyze the frequency of 8 mutations (F508del, G542X, R1162X, N1303K, W1282X, G85E, 3120+1G>A, and 711+1G>T) in a sample of 111 newborn patients with cystic fibrosis diagnosed by the Cystic Fibrosis Neonatal Screening Program of Minas Gerais State. The mutations were tested by allele-specific oligonucleotide PCR with specially designed primers. An allele frequency of 48.2% was observed for the F508del mutation, and allele frequencies of 5.41, 4.50, 4.05, and 3.60% were found for the R1162X, G542X, 3120+1G>A, and G85E mutations, respectively. The genotypes obtained were in Hardy-Weinberg equilibrium. These data demonstrate that the 8-mutation panel studied here has extensive coverage (68%) for the cystic fibrosis mutations in Minas Gerais. These data improve our knowledge of cystic fibrosis in Brazil, particularly in this region. In addition, this investigation contributed to the establishment of a sensitive and population-specific mutation panel, which can be helpful for molecular diagnosis of cystic fibrosis.

Key words: CFTR gene; Cystic fibrosis; Mutations; Neonatal screening; F508del

Introduction

Cystic fibrosis (CF) is one of the most common autosomal recessive diseases in the world (CF; MIM# 219700). This condition is caused by mutations in the CF transmembrane conductance regulator gene (CFTR) (1,2). At present, more than 1500 mutations have been identified in CF patients (3). The most common CFTR mutation is a 3-bp deletion located in exon 10 named F508del. This mutation produces a phenylalanine deletion at position 508 of the protein. The F508del mutation is distributed worldwide, but is more prevalent among Caucasians, reaching a frequency of 80% in some Western European countries (4). Other mutations such as G542X, G551D and N1303K are commonly found throughout the world, depending on geographical and ethnic features (5). In Latin America, the frequency of the F508del mutation ranges from 59% (Argentina) to 23% (Costa Rica). Other common mutations in this region are G542X, N1303K, W1282X, and R1162X (6).

Several studies analyzing the spectrum of CFTR mutations have been carried out in Brazil. Some special characteristics of this country are its geographical dimension and the ethnic variability of different regions. According to a recent study, the average estimate of the F508del frequency calculated for five populous Brazilian states is close to 48% (7). Several studies using different molecular biology methods have been published in Brazil showing the mutation profile in some states or regions (8-17). Despite the large variations in ethnic background among different regions, G542X, N1303K and R1162X have been considered to be the most frequent non-F508del mutations in Brazil (6,11,13).

The State of Minas Gerais has a population of mixed ethnic composition of European and African ancestries in a population of about 19 million people (18). A public neonatal screening program for CF (CFNS) was initiated in this state in 2003 under the responsibility of "Núcleo de Ações e Pesquisa em Apoio Diagnóstico" (NUPAD), Universidade Federal de Minas Gerais. Since July 2003, CF has been detected in 111 newborn children by this program, and consequently an estimated CF prevalence of 1:10.000. The objective of the present investigation was to determine the frequency of 8 CFTR mutations (G85E, 711+1G>T, F508del, G542X,

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3120+1G>A, R1162X, W1282X, and N1303K) in 111 sweat test-positive newborns screened by the CFNS program in the State of Minas Gerais, Brazil.

Material and Methods

A total of 111 samples from unrelated newborns screened by the CFNS program of Minas Gerais, Brazil, were studied. Neonatal screening for CF in this state is performed by a two-stage protocol: the initial test is the measurement of immunoreactive trypsinogen in dried spot samples, followed by a second assay for babies with immunoreactive trypsinogen ≥70 ng/mL. All newborns with a positive screening test are confirmed by sweat chloride testing. The 111 CF cases studied here were the total number of CF patients detected between July 2003 and April 2008 by the mandatory neonatal screening program. Therefore, no filter for any geographic or ethnic background was used in this investigation. In all patients, the diagnosis of CF was confirmed on the basis of both the sweat chloride test concentrations (>60 mEq/L) and clinical findings. Sweat chloride results ranged from 64 to 152 mEq/L. No false-negative cases were reported by the CFNS program. Age at diagnosis ranged from 30 to 40 days. Dried blood samples were collected on filter paper and sent to “Laboratório de Genética e Biologia Molecular” of NUPAD at Universidade Federal de Minas Gerais.

A preliminary analysis was performed on dried spot samples of 32 unrelated newborns referred by the CFNS program of Minas Gerais with a diagnosis of CF confirmed by the screening of 29 CF mutations. No geographic or continental group (Euro-Brazilian or African-Brazilian) selection was performed for these 32 CF patients. Mutation analysis was carried out with the Elucigene® CF kit (Tepnel Life Sciences PLC, UK). All mutations detected in this previous analysis have been included in the present investigation. The mutations are: G85E, 711+1G>T, F508del, G542X, 3120+1G>A, R1162X, W1282X, and N1303K.

In the present study, the eight mutations listed above were tested by the allele-specific oligonucleotide polymerase chain reaction (ASO-PCR) using primers designed for specific pairing with mutant and normal alleles (Table 1).

Table 1. Primers used for allele-specific oligonucleotide polymerase chain reaction.

Mutation	Primer sequence (5' → 3')	Amplicon size (bp)	Annealing temperature (°C)
G85E-S	GGA GAT TTA TGT TCT ATG G	245	52
G85E-M	GGA GAT TTA TGT TCT ATG A		
G85E-R	GTA AAT TGC CAC CCG TGT TCC AGG		
711+1G>T-S	CCA ACA ACC TGA ACA AAT TTG ATG AAG	340	64
711+1G>T-M	CCA ACA ACC TGA ACA AAT TTG ATG AAT		
711+1G>T-R	TTG CTC AGG TAT CAT ATC TGG CC		
F508del-S	ACC ATT AAA GAA AAT ATC ATC TT	262	54
F508del-M	ACC ATT AAA GAA AAT ATC ATT GG		
F508del-R	TGC AAG CTT CTT AAA GCA TA		
G542X-S	GCA GAG AAA GAC AAT ATA GTT CTT G	213/217	58
G542X-M	GTT TGC AGA GAA AGA CAA TAT AGT TCT TTT		
G542X-R	CCA CTA GCC ATA AAA CCC CAG G		
3120+1G>A-S	CTT ACC ATA TTT GAC TTC ATC CAG G	191	62
3120+1G>A-M	CTT ACC ATA TTT GAC TTC ATC CAG A		
3120+1G>A-R	TTA CTA AAC TTA TGT CTA TTT TGA AGG C		
R1162X-S	TTA TTT CAG ATG CGA TCT GTG AGC C	117	63
R1162X-M	TTA TTT CAG ATG CGA TCT GTG AGC TT		
R1162X-R	AAT CAT AAC TTT CGA GAG TTG GCC		
W1282X-S	GGG ATT CAA TAA CTT TGC AAC AGT GG	203	67
W1282X-M	GGG ATT CAA TAA CTT TGC AAC AGT GA		
W1282X-R	TCT GCC TAT GAG AAA ACT GCA CTG GAG		
N1303K-S	TTT TTT CTG GAA CAT TTA GAA AAA AC	137	58
N1303K-M	TTT TTT CTG GAA CAT TTA GAA AAA AG		
N1303K-R	GCC ATT TGT GTT GGT ATG AGT TAC CCC		

The -S suffix indicates a wild allele specific primer, the -M suffix a mutant allele primer, and the -R suffix the primer used in both wild and mutant allele amplification.

DNA was extracted using the Chelex-100 method, as described (19). ASO-PCR was carried out in a final volume of 25 µL containing 0.2 mM dNTPs, 10 mM Tris-HCl, pH 8.3, 3 mM $MgCl_2$, 10 pmol of each primer, 1.25 U GoTaq® DNA polymerase (Promega Corp., USA) and 100 ng DNA. Amplification was carried out in a GeneAmp 9700 thermocycler (Applied Biosystems, USA). Thermal conditions were: 5 min at 94°C followed by 35 cycles of 1 min at 95°C, 30 s at the specific annealing temperature (see Table 1), and 30 s at 72°C, with a final extension step for 10 min at 72°C. The structures of the primers used are shown in Table 1. All PCR products, positive, negative and reaction controls were electrophoresed on a 6% acrylamide gel for 20 min and visualized by silver staining.

Allele frequencies were estimated from the number of genotypes observed in the 111 samples analyzed (gene-counting method). A possible deviation from Hardy-Weinberg equilibrium (HWE) was tested for the chi-square goodness of fit test and the Monte-Carlo Markov Chain exact test (20) using the GENEPOP program (21). The chi-square test was used in order to compare the present frequencies with other CF mutation frequencies observed in other Brazilian populations.

Results

The present study permitted the characterization of 151 (68%) of 222 CF chromosomes analyzed. The complete genotype was determined in 52 (47%) patients and was characterized in at least one allele in 99 (89%) cases. No mutation was identified in 11% of cases.

Twenty-nine patients were homozygous for the F508del mutation (26%), 21 patients were compound heterozygotes for F508del and another mutation identified, whereas 28 were partially characterized only for F508del. Moreover, only one patient was homozygous for the R1162X mutation, whereas another one was compound heterozygous for the R1162X and 3120+1G>A mutations. Finally, 19 patients were partially characterized for non-F508del mutations. There were no significant deviations from HWE for the overall genotypes obtained. Observed and expected genotypes and statistical parameters are shown in Table 2.

The F508del mutation was detected in 107 of 222 CF chromosomes (48%). R1162X, G542X, 3120+1G>A, and G85E were the most frequent non-F508del mutations observed, comprising 12 (5.4%), 10 (4.5%), 9 (4.1%), and 8 (3.6%) of the chromosomes studied. N1303K, 711+1G>T and W1282X mutations had frequencies of less than 1%. The relative frequencies of these eight CF mutations are shown in Table 3.

Table 2. Genotypes of 111 cystic fibrosis patients from Minas Gerais, Brazil.

Genotype	Observed	Expected
F508del / F508del	29 (26.13%)	25.66 (23.12%)
F508del / ?	28 (25.23%)	34.38 (30.97%)
F508del / G542X	5 (4.50%)	4.84 (4.36%)
F508del / 3120+1G>A	4 (3.60%)	4.36 (3.93%)
F508del / R1162X	4 (3.60%)	5.81 (5.23%)
F508del / G85E	4 (3.60%)	3.87 (3.49%)
F508del / 711+1G>T	2 (1.80%)	0.97 (0.87%)
F508del / W1282X	1 (0.90%)	0.48 (0.43%)
F508del / N1303K	1 (0.90%)	0.97 (0.87%)
G542X / ?	5 (4.50%)	3.21 (2.89%)
R1162X / ?	5 (4.50%)	3.86 (3.48%)
G85E / ?	4 (3.60%)	2.57 (2.32%)
3120+1G>A / ?	4 (3.60%)	2.89 (2.60%)
3120+1G>A / R1162X	1 (0.90%)	0.49 (0.44%)
R1162X / R1162X	1 (0.90%)	0.30 (0.27%)
N1303K / ?	1 (0.90%)	0.64 (0.58%)
? / ?	12 (10.81%)	11.24 (10.13%)
Non-observed	0 (0.00%)	4.46 (4.02%)
Total	111 (100.00%)	111.00 (100.00%)
HWE (χ^2)	13.3 (d.f. = 17); P = 0.715	
HWE (MCMC)	P = 0.9438; S.E. = 0.0089	

Data are reported as number with percent in parentheses. Expected = the expected number based on the Hardy-Weinberg equilibrium (HWE). MCMC = Monte-Carlo Markov chain exact test. ? = unknown allele. d.f. = degrees of freedom.

Table 3. Allele frequencies of 8 transmembrane conductance regulator gene (CFTR) mutations in 111 cystic fibrosis patients from Minas Gerais, Brazil.

Mutation	N	Frequency (%)	Cumulative frequency (%)
G85E	8	3.60	3.60
711+1G>T	2	0.90	4.50
F508del	107	48.20	52.70
G542X	10	4.50	57.20
3120+1G>A	9	4.05	61.25
R1162X	12	5.41	66.66
W1282X	1	0.45	67.11
N1303K	2	0.90	68.01
Unknown alleles	71	31.99	
Total	222	100.00	100.00

N = number of observed alleles.

Discussion

Our sample group consisted of all CF cases detected in the State of Minas Gerais by the CFNS program between 2003 and 2008. As explained above, no ethnic or geographical limitations were applied in this investigation. Therefore, the data obtained in the present study are representative of the ethnic admixture of the population and are directly related to the incidence of CF in this region. On the other hand, the panel used was defined from a group of unclassified CF patients; therefore, we assumed that this mutation panel would be useful for the general population of Minas Gerais, rather than a specific ethnic group.

As far as we know, this is the first frequency analysis of the main CF mutations performed in Brazil in patients detected by neonatal screening. Sampling by neonatal screening considerably reduced the bias in mutation frequency calculation when compared with sampling of patients detected by typical clinical diagnosis. In the latter case, individuals with more severe genotypes (i.e., F508del homozygous) could die before diagnosis; therefore, these genotypes could be sub-represented in the sampling. On the other hand, neonatal screening is able to detect cases with milder phenotypes, which are difficult to detect by clinical diagnosis; this also contributes to a more refined estimate of CF mutation frequencies (7).

Significant agreement with the HWE was observed in the genotypic data. In previous studies carried out on patients detected by clinical diagnosis, a significant departure from the HWE has been found, possibly due to the influence of the morbimortality of some genotypes, specifically F508del homozygotes (16). As considered above, this effect must have been smaller in our group due to the young age of the patients (30-40 days). In addition, the agreement with HWE observed was also remarkable, considering the ethnic heterogeneity of the sample.

The frequency of F508del in our sample was 48%, similar to that found in the Southern region of Brazil ($P > 0.90$) (14) but different from that reported in studies from São Paulo, Rio de Janeiro or the Northern region ($P < 0.005$) (8,9,15). In Minas Gerais, Raskin et al. (7,13) reported a similar F508del allele frequency ($P > 0.90$) but only patients of European descent were studied. Although the G542X mutation has been reported to be the most frequent non-F508del mutation in most Brazilian regions, R1162X mutation shows a higher frequency than G542X in the State of Minas Gerais. This result is not surprising considering the few data available on R1162X mutation in Brazil. On the other hand, previous studies have observed that R1162X was the second most frequent mutation in the State of Santa Catarina, as well as in Minas Gerais (in African-Brazilians patients) (13). The frequency for G542X (4.5%) was lower than that reported for São Paulo and for other regions in the south of the country (8,10,11,13,17).

The present investigation revealed that the 8-mutation panel studied has an extensive coverage rate (68%) for CFTR mutations in Minas Gerais. Previous studies carried out in Brazil using multimutation panels have reported a coverage rate of 26% for 4 mutations in the North (15), 42.5% for 5 mutations in São Paulo (8), and 54.5% for 11 mutations in the South (14). A recent study conducted in the south of Brazil involving a complete scanning of the CFTR gene detected 95% of mutated alleles in Santa Catarina State and 73% of CF alleles in Paraná State (17). In a previous study in Minas Gerais, a coverage rate of 75% was reported for 13 mutations in Euro-Brazilian patients and a coverage rate of 21% was reported for 7 mutations in Afro-Brazilian patients (13). Moreover, a recent compilation of CFTR mutations found in Latin-American countries indicated that a "minimum panel" covering the six most frequent mutations reported in Brazil (F508del, G542X, R1162X, W1282X, N1303K, and R334W) would have an estimated detection power of 53% (6). We would expect that the addition in the future of other mutations already observed in this country (i.e., R334W, R553X, G551D, 1717-1G>A, or 1812-1G>A) should significantly increase the detection power of this panel. Moreover, although 3120+1G>A is already included in our panel, other mutations of African origin, like G1249E, A559T or S1255X, could be investigated and added in the future (22). Recent studies have underscored the necessity to apply well-defined mutational arrays, which are important for ethnically admixed populations or in areas with a low frequency of F508del (5,23). In countries with a high ethnic heterogeneity, like Brazil, it is necessary to have region-specific mutation panels, permitting an increase of sensitivity of the panel for diagnostic purposes and for CF screening programs. In this context, the fact that the panel presented here detected at least one mutated allele in 89% of 111 patients analyzed leads one to consider, in theory, their application in a neonatal screening protocol. Nevertheless, all protocols for CFNS have different advantages and drawbacks, including practical, economical and logistic issues (24).

In conclusion, the present study improves the knowledge of CF in Brazil and in the State of Minas Gerais in particular. In addition, this investigation contributes to establishing a sensitive and population-specific mutation panel, which can be helpful for the molecular diagnosis of CF in this region.

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