

# Molecular Analysis in Brazilian Cystic Fibrosis Patients Reveals Five Novel Mutations

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## ABSTRACT

We have performed molecular genetic analyses on 160 Brazilian patients diagnosed with cystic fibrosis (CF). Screening of mutations in 320 CF chromosomes was performed through single strand conformation polymorphism (SSCP) and heteroduplex analyses assay followed by DNA sequencing of the 27 exons and exon/intron boundaries of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. The frequency of CFTR variants of T-tract length of intron 8 (IVS8 Tn) was also investigated. This analysis enabled the detection of 232/320 CF mutations (72.2%) and complete genotyping of 61% of the patients. The  $\Delta$ F508 mutation was found in 48.4% of the alleles. Another fifteen mutations (previously reported) were detected: G542X, R1162X, N1303K, R334W, W1282X, G58E, L206W, R553X, 621+1G→T, V232D, 1717-1G→A, 2347 delG, R851L, 2789+5G→A, and W1089X. Five novel mutations were identified, V201M (exon 6a), Y275X (exon 6b), 2686 insT (exon 14a), 3171 delC (exon 17a), and 3617 delGA (exon 19). These results contribute to the molecular characterization of CF in the Brazilian population. In addition, the identification of the novel mutation Y275X allowed prenatal diagnosis in a high-risk fetus.

## INTRODUCTION

CYSTIC FIBROSIS (CF) is the commonest severe autosomal recessive disorder in the Caucasian populations, with an estimated incidence of 1 in 2,500 live births and a carrier frequency of 1 in 25 (Boat *et al.*, 1989). The main clinical manifestations include chronic obstructive pulmonary disease, pancreatic insufficiency and associated gastrointestinal problems, meconium ileus, and elevated electrolyte concentration in the sweat. The cystic fibrosis transmembrane conductance regulator (CFTR) gene at chromosome 7q31 has 27 exons spanning 230 kb and encodes a protein of 1,480 amino acids (Kerem *et al.*, 1989; Riordan *et al.*, 1989; Rommens *et al.*, 1989).

More than 800 different mutations in the CFTR gene have been reported to the Cystic Fibrosis Genetic Analysis Consortium so far. The frequency of these mutations varies in different populations. The most common mutation is a 3-bp deletion that results in the loss of a phenylalanine at position 508

( $\Delta$ F508). In Europe, the frequency of this mutation varies in different populations and geographic areas, forming a northwest to southeast gradient. There is little information about the frequency of  $\Delta$ F508 and other CF mutations in Brazilian or other Latin American populations (Orozco *et al.*, 1993; Raskin *et al.*, 1993; Chertkoff *et al.*, 1997; Parizotto *et al.*, 1997; Vilalobos-Torres *et al.*, 1997; Rabbi-Bortolini *et al.*, 1998).

The ethnic origin of the Brazilian population is very heterogeneous as a result of a variety of migratory currents irregularly distributed in Brazilian regions. Besides the Portuguese and Africans, about 4.5 millions immigrants, mainly from Italy, Germany, Spain, and Japan, have come to Brazil (Barreto, 1959).

The determination of the type and frequency of CF mutations in such a mixed population is extremely interesting. In addition, studies on genotype/phenotype correlations are very important for prognosis and effective genetic counseling. Here we report the result of molecular analysis of the entire CFTR cod-

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ing sequence, including exon/intron boundaries in 160 CF patients (320 chromosomes). We have also determined the frequency of CFTR variants of T-tract length of intron 8 (IVS8 Tn) (which are associated with varying degrees of exon 9 splicing), to assess any association with atypical CF expression.

This is the first study to report the screening of the entire coding region of the CFTR gene in the Brazilian population. A total of 21 pathogenic mutations, including five novel mutations and five polymorphisms, were found in Brazilian patients.

## PATIENTS AND METHODS

### Sample

A total of 160 Brazilian CF patients (54% males, 46% females) were analyzed. These patients were referred to the Centro de Estudos do Genoma Humano (Center for the Studies of the Human Genome), at the University of São Paulo, with a diagnosis of CF. The diagnosis of CF was based on clinical findings (pulmonary and/or pancreatic involvement) together with elevated sweat chloride levels (>60 mEq/liter of chloride). In 2 patients in whom it was not possible to perform iontophoresis sweat testing, the clinical symptoms strongly suggested CF. Blood from patients and their family members was collected in vacutainers with EDTA as anticoagulant, after informed consent.

### DNA analysis

Mutation detection studies were carried out in all 27 exons and exon/intron boundaries of the CFTR gene through single strand conformation polymorphism (SSCP; Orita *et al.*, 1989) and heteroduplex analysis. A 100-ng sample of genomic DNA was amplified under the following conditions: 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM of each primer, and 2 units of *Taq* polymerase (Gibco BRL) for 35 cycles. All PCR reactions were performed in 10  $\mu$ l total.

The different conditions for performing SSCP/heteroduplex analysis were as reported by Ravnik-Glavac *et al.* (1994). Abnormally migrating fragments were subsequently sequenced to confirm the presence of mutations.

The CFTR gene variants of T-tract length of intron 8 were determined in the 160 patients. The analysis of the microsatellite IVS8 (T<sub>n</sub>) was made by PCR, and the amplified fragments were subjected to electrophoresis at 1500 V for 4 hours in a 6.5% denaturing polyacrylamide gel.

## RESULTS

### Families

Among the 160 CF patients included in the present study, 15 were born from consanguineous parents.

The ethnic distribution showed that 117 (73%) were Caucasian (from European descent) and 43 (27%) were of mixed racial background (Caucasian, Neegroid, and/or native indigenous). This mixed group was classified as Brazilian.

### Mutation screening

The 320 CF chromosomes were first analyzed by SSCP/heteroduplex analysis. All abnormally migrating bands were subsequently sequenced. A mutation was identified in 231 (72.2%) of the CF chromosomes and the complete genotyping was achieved in 97 CF patients (Table 1).

The  $\Delta$ F508 mutation was present in 48.4% of the CF chromosomes. Forty-seven patients (29.4%) were  $\Delta$ F508 homozygotes, 61 (38.1%) were compound heterozygotes for  $\Delta$ F08 and another mutation, and 51 (32.5%) carried two non- $\Delta$ F508 mutations. The next most common mutations were: G542X (8.8%), R1162X (2.5%), N1303K (2.5%), R334W (2.5%), W1282X (1.3%), G58E (1.3%), L206W (0.6%), and R553X (0.6%). These mutations account for about 20.1% of the non- $\Delta$ F508 alleles.

Seven other rare mutations were also identified: 621+1G $\rightarrow$ T (exon 4), V232D (exon 6a), 1717-1G $\rightarrow$ A (intron 11), 2347 delG (exon 13b), R851L (exon 14a), 2789+5G $\rightarrow$ A (intron 14b), and W1089X (exon 17b). The patient homozygous for the 2347 delG mutation was born from consanguineous parents. In addition, the following polymorphisms (previously reported) were also found: M470V (exon 10), 3030 G/A (exon 15), 4002 A/G (exon 20), 4504 C/T (exon 24), and 4521 G/A (exon 24).

Five previously undescribed mutations were found and are briefly described below.

**3617 delGA:** This frameshift mutation, caused by a dinucleotide (GA) deletion at positions 3,617 and 3,618 of the coding region, creates a stop signal at codon 1,164 in exon 19. It was found in a 1-year-old female patient who is a compound heterozygote, also inheriting the  $\Delta$ F508 mutation. The patient has a severe phenotype with pancreatic insufficiency, chronic lung disease, and a high sweat test score.

**3171 delC:** This frameshift mutation, caused by a C deletion at position 3,171 of the coding region, creates a stop signal at codon 1,022 in exon 17a. The other chromosome carries a  $\Delta$ F508 mutation. The patient, a 6-year-old male at ascertainment, was diagnosed at 4 months. Clinical symptoms include moderate pulmonary disease, pancreatic insufficiency, and a high sweat test score.

**2686 insT:** This frameshift mutation, caused by a T insertion after position 2,686, creates a stop signal at codon 885 in exon 14a. The patient, a 2-year-old compound heterozygote female, also inheriting  $\Delta$ F508, was diagnosed at 2 months old; she has a high sweat test score, pancreatic insufficiency, and frequent pulmonary infections.

**Y275X:** This G $\rightarrow$ C transition was detected at position 957 in exon 6b, resulting in the replacement of a tyrosine (position 275) by a termination codon. The other chromosome carried a  $\Delta$ F508 mutation. It was identified in a male patient who presented with a severe CF phenotype and died at the age of 6 months.

**V201M:** This G $\rightarrow$ A transition, at position 733 in exon 6a, leads to the substitution of a valine for a methionine. This mutation was detected in a 8-year-old male, diagnosed at age 6. This patient has mild pulmonary disease, a high sweat test score, and no pancreatic insufficiency. The second mutation is still unknown.

TABLE 1. GENOTYPES, FREQUENCIES, AND PRESENCE OF PI FROM 160 CF PATIENTS (320 CF CHROMOSOMES)

Genotype	Number	Frequency (%)	Number and frequency (%) of patients with PI
ΔF508/ΔF508	47	29.40	47 (100%)
ΔF508/G542X	13	8.10	13 (100%)
ΔF508/R1162X	6	3.80	6 (100%)
ΔF508/R334W	5	3.10	3 (60%)
ΔF508/N1303K	3	1.90	3 (100%)
ΔF508/W1282X	2	1.20	2 (100%)
ΔF508/G58E	2	1.20	1 (50%)
ΔF508/L206W	1	0.62	0
ΔF508/R553X	1	0.62	1 (100%)
ΔF508/R851L	1	0.62	0
ΔF508/2789 + 5g → A	1	0.62	0
ΔF508/3617delGA	1	0.62	1 (100%)
ΔF508/3171delC	1	0.62	1 (100%)
ΔF508/2686insT	1	0.62	1 (100%)
ΔF508/Y275X	1	0.62	1 (100%)
ΔF508/U	22	13.80	14 (64%)
G542X/G542X	3	1.90	3 (100%)
G542X/N1303K	3	1.90	2 (67%)
G542X/R1162X	1	0.62	1 (100%)
G542X/U	5	3.10	4 (80%)
N1303K/R1162X	1	0.62	1 (100%)
N1303K/G58E	1	0.62	0
2347delG/2347delG	1	0.62	1 (100%)
R334W/V232D	1	0.62	0
R334W/W1089X	1	0.62	1 (100%)
R334W/U	1	0.62	1 (100%)
W1282X/U	1	0.62	1 (100%)
G58E/U	1	0.62	1 (100%)
R553X/U	1	0.62	1 (100%)
L206W/U	1	0.62	0
621 + 1G → T/U	1	0.62	1 (100%)
1717-1G → A/U	1	0.62	Not known
V201M/U	1	0.62	0
U/U	27	16.90	12 (44%)
Total	160	100	—

U, Unknown CF mutation.

### Analysis of IVS8-Tn tract genotype

The analysis of the polythymidine tract at the splice acceptor site of intron 8 of the 320 CF chromosomes showed the following distribution: 229 (71.6%) 9T alleles, 88 (27.5%) 7T, and 3 (0.9%) 5T alleles.

## DISCUSSION

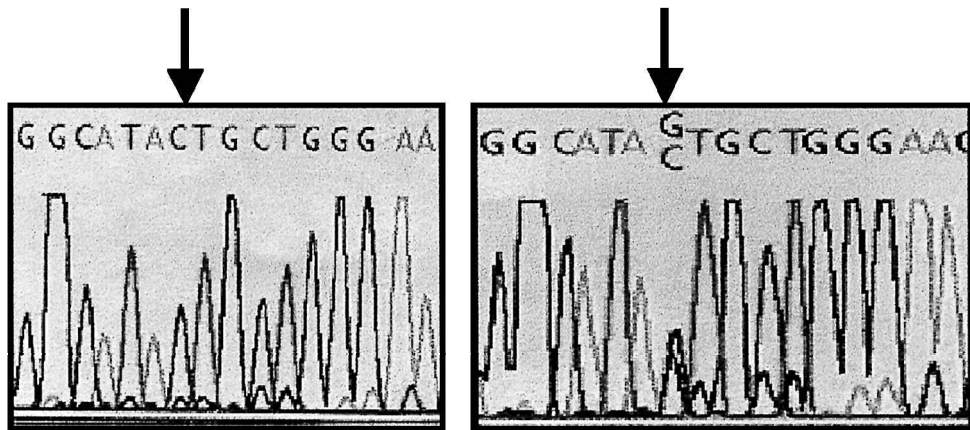
More than 800 mutations have been reported to the Cystic Fibrosis Genetic Analysis Consortium. The frequency of each of these mutations varies according to the population under study.

Here, a total of 320 chromosomes were analyzed for the entire coding sequence of the CFTR gene, including exon-flanking intron sequences. In 160 patients, the diagnosis was based on typical clinical manifestation and high or borderline sweat

electrolyte concentration. Through SSCP/heteroduplex analysis and sequencing, 72.2% of the CF mutations were identified. The 27.8% undetected mutations might have escaped detection because of the limited sensitivity of the techniques used, or due to mutations outside the coding regions or in the promoter region.

Previous reports have shown that CF mutations are distributed over the whole CFTR gene (Tsui, 1992; Ferec *et al.*, 1992; Cheadle *et al.*, 1993). In this study, 16 mutations were identified: ΔF508, G542X, R1162X, N1303K, R334W, W1282X, G58E, L206W, R553X, 621+1G→T, V232D, 1717-1G→A, 2347 delG, R851L, 2789+5G→A, and W1089X. These mutations correspond to 13 of the 27 CFTR exons, or about 50% of the gene.

Several studies have shown a considerable variation in the distribution of the ΔF508 mutation in different populations, with frequencies of 87% in the Danish (Schwartz *et al.*, 1990), 80% in the British (Watson *et al.*, 1990), 55% in the Italians



**FIG. 1.** Mutation Y275X. Sequence analysis in an automatic sequencer (Perkin-Elmer ABI model 310) demonstrating a C (left side) to a G substitution (right side) at amino acid position 247, resulting in a premature stop codon.

(Novelli *et al.*, 1990), 50% in the Spanish (Estivill *et al.*, 1989), and 30% in Ashkenazi Jews (Lemna *et al.*, 1990). In the present sample, the observed frequency (48.4%) of the most common mutation,  $\Delta F508$ , is similar to the value found in a previous report of Brazilian CF patients (47%) (Raskin *et al.*, 1993). This finding is consistent with the ethnic composition of our country, which is predominantly of immigrants from Portugal, Africa, Italy, and Spain. The second most common mutation, G542X, which occurred in 8.8% of our patients, is also the second most frequently reported in Spanish Mediterranean coastal areas (8.0%) (Casals *et al.*, 1993).

Two other mutations—N1303K (2.5%) and R1162X (2.5%)—were also found in frequencies compatible with the ethnic origins of our Caucasian population. The N1303K mutation has been reported in Southern Europeans as the fourth most common mutation, with a frequency of 3.2% (Nunes *et al.*, 1991), and the R1162X mutation is the second most frequent mutation in Northern Italy (about 10%) (Casals *et al.*, 1993). However, the R334W mutation, which we found in 8 patients, is apparently more frequent in our population (2.5%) than the 0.4% frequency reported worldwide (Tsui, 1992). On the other hand, the 3120+1G→A mutation, which has been reported in African-American CF patients and South African blacks (Padoa *et al.*, 1999), was not observed in our patients, although 27% of them had some African ancestors. The fact that we did not observe this mutation in our population might be due to the sample size or the fact that our African-Brazilian population came from different regions of Africa.

A total of 229 CF chromosomes (71.6%) were found associated with allele 9T at locus IVS8(Tn). The absolute linkage disequilibrium found between  $\Delta F508$  and the 9T allele is in accordance with that observed in chromosomes of European descent in other series (Cuppens *et al.*, 1994; Dörk *et al.*, 1994; Claustres *et al.*, 1996). The other alleles, 7T and 5T, were present at frequencies of 27.5% and 0.9%, respectively. Previous studies have shown that 77.2% of CF alleles are associated with allele 9T and 22.8% with allele 7T (Chu *et al.*, 1993). In accordance with the literature, none of the 21 CFTR mutations identified in our patients were found to be associated with allele 5T. On the other hand, the 5T alleles have also been found

in some CF patients with very mild symptoms (Dörk *et al.*, 1994). Our 3 patients with allele 5T also have mild pulmonary disease, borderline sweat tests, and no pancreatic insufficiency.

Analysis of genotype/phenotype correlation has shown that 125 patients (78%) have pancreatic insufficiency (PI). Kristidis *et al.* (1992) suggested that pancreatic sufficiency (PS) occurs in patients who have one or two mild mutations whereas PI occurs almost always in those with two severe mutations. The majority of our patients (52%) carried two severe alleles, such as  $\Delta F508$ . However, one of our compound heterozygote patients for severe mutations (N1303K/G542X), has PS. The mutation R334W, which is considered a later-onset PI mutation, was first reported to be associated with PS (Kristidis *et al.*, 1992). In the present sample, this mutation combined with severe mutant alleles was responsible for either PS or PI. The proportion of PI patients with the R334W mutation in our sample (63%), is similar to the values found by Estivill *et al.* (1995).

The frequency of consanguineous marriages in this sample was 9.4%. Among the patients who were homozygous for CFTR mutations, 5.6%, were born from consanguineous parents. Unexpectedly, however one compound heterozygote (N1303K/G542X) CF patients (who was referred to above as having PS) was the son of first cousins, which is compatible with the high frequency of CFTR heterozygotes in the population.

The five novel CFTR mutations here described, four of which cause a premature stop codon (three frameshift and one nonsense mutation), are pathogenic mutations. All four were associated with a  $\Delta F508$  mutation as the other allele and resulted in a severe phenotype. The fifth mutation, the V201M change, is a missense mutation; however, it is probably the cause of disease in the affected individual, because it was not found in 200 normal chromosomes. Further investigations are required to determine the effect of these mutations on the function of the CFTR protein.

The identification of these five novel mutations reveals an important contribution of native alleles in the CFTR gene of Brazilian CF patients. One of them, the Y275X mutation (Fig. 1), is probably of Indian origin and was particularly important because it allowed a prenatal diagnosis in a high-risk fetus. This

case was referred to us for prenatal diagnosis because the parents had lost a son, at 6 months of age, with a severe form of CF. There was no DNA available from the deceased child. Screening of mutations revealed that the mother (of Portuguese descent) carried the  $\Delta F508$  mutation, but the mutation in the father (whose mother was Brazilian and whose father was of Indian descent) was unknown. The first DNA analysis of chorionic villus tissue revealed that the fetus had inherited the  $\Delta F508$  allele and, therefore, had a 50% chance of being affected. Because the parents did not want to risk another affected child, they considered terminating the pregnancy. However, the Y275X mutation identified in the father was not found in the fetus. Thus, the pregnancy went to term and a healthy baby was born.

In conclusion, the distribution of mutations in the CFTR alleles of the CF patients reflects the influence of the diverse ethnic admixture present in our country. In addition, the screening of mutations in Brazilian CF patients should provide useful information for diagnosis, genetic counseling, prenatal diagnosis, and the enhancement of our knowledge of genotype/phenotype correlations in this disease.

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