

High Allelic Heterogeneity Between Afro-Brazilians and Euro-Brazilians Impacts Cystic Fibrosis Genetic Testing

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ABSTRACT

Cystic fibrosis (CF) is an autosomal recessive disease caused by at least 1,000 different mutations in the cystic fibrosis transmembrane conductance regulator gene (*CFTR*). To determine the frequency of 70 common worldwide *CFTR* mutations in 155 Euro-Brazilian CF patients and in 38 Afro-Brazilian CF patients, we used direct PCR amplification of DNA from a total of 386 chromosomes from CF patients born in three different states of Brazil. The results show that screening for seventy mutations accounts for 81% of the CF alleles in Euro-Brazilians, but only 21% in the Afro-Brazilian group. We found 21 different mutations in Euro-Brazilians and only 7 mutations in Afro-Brazilians. The frequency of mutations and the number of different mutations detected in Euro-Brazilians are different from Northern European and North American populations, but similar to Southern European populations; in Afro-Brazilians, the mix of CF-mutations is different from those reported in Afro-American CF patients. We also found significant differences in detection rates between Euro-Brazilian (75%) and Afro-Brazilian CF patients (21%) living in the same state, Minas Gerais. These results, therefore, have implications for the use of DNA-based tests for risk assessment in heterogeneous populations like the Brazilians. Further studies are needed to identify the remaining CF mutations in the different populations and regions of Brazil.

INTRODUCTION

CYSTIC FIBROSIS (CF) is the most common lethal genetic disorder among Caucasians. In 1989, the gene responsible for CF, referred to as the cystic fibrosis transmembrane conductance regulator (*CFTR*), was cloned. The most common *CFTR* mutation is a 3-bp deletion that causes the loss of the phenylalanine residue at position 508 ($\Delta F508$) in its tenth exon (Riordan *et al.*, 1989; Rommens *et al.*, 1989; Kerem *et al.*, 1989). Although the major mutation causing CF accounts for 66% of mutant chromosomes screened worldwide, at least 1,000 sequence alterations associated with the disease have been identified in the *CFTR* gene during the past years, and their fre-

quencies vary between populations (Tsui, 1990, 1992; Cystic Fibrosis Genetic Analysis Consortium, 1994, 1999). Previously, we have shown allelic heterogeneity in Brazilian CF patients of European origin by screening for $\Delta F508$ and another four common worldwide mutations (G542X, N1303K, G551D, and R553X). These five mutations represent 56% of Euro-Brazilians CF alleles, and their frequencies, as well as the associated haplotypes, vary from state to state (Raskin *et al.*, 1993, 1997a,b, 1999).

Brazil is a country larger in size than all of Western Europe, and larger than the United States if Alaska is excluded. With a population of approximately 170 million persons (IBGE, 2002), Brazil is the sixth most populous country in the world. Brazil

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was conquered by Portugal in 1500, but various other European, Middle Eastern, and Asian immigrant groups have settled in Brazil since the mid-19th century. An important migration flow came from Europe, starting in 1802 and decreasing dramatically after 1950. From 1875 until 1950, about 5 million Europeans immigrated to Brazil, settling mainly in the southern states. Eighty percent of the immigrants have come from Portugal, Italy, Spain, and Germany. Africans were brought to Brazil as slaves during the 16th to the 19th century.

Although the European immigrant population was heterogeneous and admixture (with the natives and African immigrants) was widespread, European descendants relatively free from admixture are found, primarily in the South: for example, in the states of Parana (PR) and Santa Catarina (SC) (Salzano and Freire-Maia, 1967; Salzano and Pena, 1987; Pereira *et al.*, 1999). SC is the Brazilian state with the lowest admixture rate between Euro and Afro descendants (7%); its current population is made up of 90% Euro-Brazilians and only 2.2% of nonmixed Afro-Brazilians. The population of PR is made up of 78% Euro-Brazilians and 3% of nonmixed Afro-Brazilians (IBGE, 2002). Intermarriage between the Portuguese and indigenous people or African slaves was very common, and, therefore, the five large regions of Brazil (North, Northeast, Southeast, South, and West-Central) vary in percentage of European background. Today, Euro-Brazilians make up 53% of the total population; Afro-Brazilians are 38.9%, nonmixed Afro-Brazilians 6%, and Brazilians of Asian origin 0.5%. Indigenous full-blooded Indians, located mainly in the northern and western border regions and in the upper Amazon Basin, constitute less than 1% of the population (IBGE, 2002). Therefore, the current Brazilian population is composed mainly of a dihybrid population in the South and Southeast (descendants of Europeans and/or Africans), and by a trihybrid admixture (of Europeans, Africans, and/or Indians) in other regions, mainly the North and Northeast.

The state of Minas Gerais (MG), in the southeast region, received one of the largest concentrations of African slaves, and is where the most intense admixture between Brazilians of European and African origin occurred. In MG, there is a high proportion of Afro-Brazilians (38%), when compared to PR and SC (17% and 7%, respectively). In MG, the rest of the population is made up of 54% Euro-Brazilians and 7.3% nonmixed Afro-Brazilians (IBGE, 2002).

To determine the frequency of 70 *CFTR* mutations in Brazilian CF patients of European and African origin born in three different states of Brazil, 386 chromosomes from 193 Brazilian CF patients were screened by direct PCR amplification of DNA.

MATERIALS AND METHODS

Subject population

After informed consent from the families, we studied 193 nonrelated Brazilian CF subjects selected from three different states of Southeast and Southern Brazil (48 subjects were born in SC, 50 in PR, and 95 in MG). Each of these three states is served by well-established regional CF reference centers. Fifty-four percent of the subjects were male and the mean age was

6.7 years, ranging from 2 months to 32 years. All of the patients and their parents were born in Brazil. From the initial MG sample of 95 CF subjects, 38 showed phenotypic signs of African descent, as described by Krieger *et al.* (1965), Azevedo (1980), and Azevedo *et al.* (1982), and, therefore, were considered to be African-Brazilians. Fifty-seven CF patients were phenotypically Caucasians and for each of them, both parents were not aware of any African ancestry in their heritage: they were, therefore, considered to be Euro-Brazilians. Based on the same criteria, all 98 patients born in SC and PR were considered to be Euro-Brazilians.

Criteria for diagnosis of CF

Criteria for diagnosis included clinical findings of chronic pulmonary disease and a positive sweat test. The clinical evaluation was carried out from 1990 to 2000 in CF reference centers.

Blood samples

Blood samples were collected by venipuncture or by fingerstick on Guthrie cards (Raskin *et al.*, 1992a,b).

DNA analysis

Samples were tested for the 70 *CFTR* mutations listed in Table 1. DNA was extracted using high salt extraction (Lahiri and Schnabel, 1993), or DNA was directly obtained from dried blood spots on Guthrie cards (Raskin *et al.*, 1992a,b). Methods were essentially as described in Shuber *et al.* (1997) and Heim *et al.* (2001).

Statistical methods

Several comparisons were performed to calculate the statistical significance of the differences in *CFTR* mutation frequency between three Brazilian states (MG, PR, and SC); between Euro- and Afro-Brazilians living in the same state (MG); and between Brazil and other countries, using Fisher's exact test.

RESULTS

Table 1 shows the frequencies of the 70 *CFTR* mutations in the three different states of Brazil and in the two continental groups.

The analysis of 386 CF chromosomes from patients born in different states of Brazil shows that only 11 mutations have relative frequencies higher than 1% and another 10 mutations are found in 0.3–0.9% of all CF chromosomes studied. Only 7 among 70 *CFTR* mutations screened were present in CF patients born in three different states of Brazil. Fourteen mutations were present only in Euro-Brazilian CF patients, and none of the 70 mutations screened were found only in the Afro-Brazilian group. In 31.1% (120/386) of the CF chromosomes, none of the 70 mutations screened could be detected.

Only three mutations have an overall frequency higher than 4%— $\Delta F508$, G542X, and R1162X—and a fourth, 3120+1G \rightarrow A, has a frequency of 2.6% in the Afro-Brazilian group. Our data show that the estimated overall prevalence of the $\Delta F508$ mutation among CF alleles from the three Brazilian states un-

TABLE 1. FREQUENCIES OF 70 *CFTR* MUTATIONS IN DIFFERENT STATES OF BRAZIL, BY CONTINENTAL GROUP

<i>CFTR</i> mutations detected	Euro-Brazilians <i>CF</i> Alleles					African-Brazilians <i>CF</i> alleles		Total of Brazilian <i>CF</i> alleles	
	<i>SC</i>	<i>PR</i>	<i>MG</i>	<i>SC, PR, MG</i> ^a		<i>n</i>	<i>%</i>	<i>N</i>	<i>%</i>
	<i>n</i>	<i>n</i>	<i>n</i>	<i>n</i>	<i>%</i>				
ΔF508	53	39	54	146	47.1	8	10.5	154	39.9
G542X	6	9	8	23	7.4	1	1.3	24	6.2
R1162X	9	2	4	15	4.8	2	2.6	17	4.4
N1303K	5	5	0	10	3.2	0	0	10	2.6
R334W	5	1	4	10	3.2	0	0	10	2.6
G85E	2	2	4	8	2.6	1	1.3	9	2.3
1717-1G→A	1	3	2	6	1.9	0	0	6	1.6
W1282X	4	1	1	6	1.9	0	0	6	1.6
3849+10kbc→T	1	3	1	5	1.6	0	0	5	1.3
R553X	0	2	0	2	0.7	0	0	2	0.5
1812-1G→A	0	1	3	4	1.3	1	1.3	5	1.3
2183AA→G	2	1	0	3	1.0	0	0	3	0.8
3120+1G→A	0	0	2	2	0.7	2	2.6	4	1.0
Y1092X	0	1	1	2	0.7	1	1.3	3	0.8
G551D	0	0	0	0	0	0	0	0	0
W1089X	0	0	1	1	0.3	0	0	1	0.3
621+G→T	0	1	0	1	0.3	0	0	1	0.3
Q1238X	0	1	0	1	0.3	0	0	1	0.3
711-1G→T	0	1	0	1	0.3	0	0	1	0.3
R347P	1	0	0	1	0.3	0	0	1	0.3
1898+1G→A	1	0	0	1	0.3	0	0	1	0.3
I507	0	0	1	1	0.3	0	0	1	0.3
Subtotal	91	73	86	250	80.7	16	21.1	266	68.9
Alleles with <i>CFTR</i> mutations not detected	5	27	28	60	19.4	60	79.0	120	31.1
Total	96	100	114	310	100.0	76	100.0	386	100.0
Detection rate (%)	94.8	73.0	75.4	250	80.7	16	21.1	266	68.9

The following 70 *CFTR* mutations were selected and tested on the basis of frequency in various populations, known association with CF, or predicted deleterious effect on the *CFTR* protein product; ΔF508, G542X, N1303K, G551D, R553X, DI507, A455E, A559T, C524X, D1270N, E60X, G178R, G330X, G85E, 2307insA, I148T, K710X, P574H, Q1238X, Q493X, Q890X, R1158X, R1162X, R117H, R334W, R347H, R347P 2307insA, I148T, K710X, P574H, Q1238X, Q493X, Q890X, R1158X, R1162X, R117H, R334W, R347H, R347P 2307insA, I148T, K710X, P574H, Q1238X, Q493X, Q890X, R1158X, R1162X, R117H, R334W, R347H, R347P, R352Q, R560T, S1196X, S1255X, S364P, S549N, S549R, V520F, W1089X, W1282X, W1310X, W1316X, Y1092X, Y122X, Y563D, 1078delT, 1677delTA, 1717-1G-A, 1812-1G-A, 1898 + 1G-A, 2043delG, 2183delAA-G, 2184delA, 2789 + 5G-A, 2869insG, 2909delT, 3120 + 1G-A, 3120G-A, 3358delAC, 3659delC, 3662delA, 3750delAG, 3791delC, 3821delT, 3849 + 10Kbc-T, 3849 + 4A-G, 3905insT, 405 + 1G-A, 444delA, 556delA, 574delA, 621 + 1G-T, and 711 + 1G-T.

^aSC, Santa Catarina State; PR, Parana State; MG, Minas Gerais State; *n*, number of chromosomes.

der investigation is 40% (154/386). Its frequency varied (55%, 39%, and 35.3%) between patients from SC, PR, and MG, respectively, but these differences are only significant between SC and PR ($p = 0.032$). The differences in ΔF508 frequency are more striking when the subjects are divided into population subgroups, Euro-Brazilians and Afro-Brazilians: The ΔF508 mutation was present in 47.1% of all 310 CF alleles examined in Euro-Brazilians, as opposed to 10.5% of 76 CF alleles examined in Afro-Brazilians ($p = 6 \times 10^{-10}$).

The detection rates varied not only between different population subgroups, but also between patients born in different states of Brazil. In SC, the detection rate was 95% by testing for only 13 mutations; in PR, the detection rate was 75% by testing for 16 mutations; and in MG, for CF patients of European origin, the detection rate was 75% by testing for 13 mutations.

DISCUSSION

The frequencies of *CFTR* mutations in the three Brazilian states studied herein varied, in agreement with previous reports showing that the frequencies of individual CF mutations can vary among different populations (Cystic Fibrosis Genetic Analysis Consortium, 1994; Estivill *et al.*, 1997). In this study, we also found that only four among 70 *CFTR* mutations included in the screening panel were present in CF patients born in the three different states of Brazil and in the two population subgroups (Euro- and Afro-Brazilians) studied; that is, ΔF508, G542X, R1162X, and G85E.

ΔF508

The data we report confirms that the ΔF508 mutation is the most frequent *CFTR* defect in the Brazilian states studied, par-

ticularly in the ones that have a high percentage of European descendants relatively free from admixture, namely SC and PR. It is quite clear that the $\Delta F508$ mutation is more frequent in Euro-Brazilian than in Afro-Brazilian CF patients, in agreement with previous work showing that $\Delta F508$ mutation first arose in the Caucasian population several hundred years ago (Morrall *et al.*, 1994; Macek *et al.*, 1997; Friedman *et al.*, 1998). Because the rate of admixture between European and African descendants in MG is around 50% (Salzano and Freire-Maia, 1967), the frequency of $\Delta F508$ in the Afro-Brazilian CF population of MG group (10.5%) could be explained almost entirely by admixture with Euro-Brazilians.

When we compare our findings to the only publication that described *CFTR* mutation screening in a large sample of CF patients of African descent (Macek *et al.*, 1997), we conclude that although in the United States and in Brazil the most common mutation is indeed $\Delta F508$ (66% and 48% in U.S. Caucasians and African-Americans, respectively, and 47% and 10.5% in Euro-Brazilians and Afro-Brazilians, respectively), our data show that the $\Delta F508$ mutation frequency in Afro-Brazilians is much lower than in Afro-Americans. The lower $\Delta F508$ frequency in Brazilian Caucasian CF patients has already been discussed in our previous work as the result of immigration into Brazil of CF carriers of southern European origin (in whom the frequency of $\Delta F508$ is lower than in northern/western Europeans) (Raskin *et al.*, 1999). The lower $\Delta F508$ frequency in Afro-Brazilian CF patients could be explained by the fact that $\Delta F508$ is lower in Euro-Brazilians (again, because of the predominance of those of southern European ancestry among Euro-Brazilians when compared to American Caucasians and, therefore, even with a higher admixture rate between those of Euro- and Afro-descent in Brazil than in United States, we would expect a lower $\Delta F508$ frequency in Afro-Brazilians as compared to Afro-Americans. The lower frequency of $\Delta F508$ can also be explained by the putative presence of other frequent mutations in the Afro-Brazilian CF gene pool, as supported by the finding of a lower number and frequency of different *CFTR* "common Caucasian mutations" in Afro-Brazilian CF patients.

G542X

Our data show that G542X is the second most common CF mutation in two of the three Euro-Brazilian CF states studied (PR and MG), with an overall frequency of 6.2% (Table 1). The presence of G542X in the Afro-Brazilian group was, as for $\Delta F508$, likely the result of Caucasian admixture, in agreement with North American data (Macek *et al.*, 1997).

R1162X

This is the third most common mutation identified to date in the Euro-Brazilian CF patients overall, and the second most frequent in SC. Its overall frequency in this sample, 4.4%, is higher than the 0.3% reported worldwide, and even higher in SC (10.4%). Mutation R1162X is more common in southern than in northern Europe (Osborne *et al.*, 1992; Cystic Fibrosis Genetic Analysis Consortium, 1994), and it has a very high frequency in those from specific regions of Italy, such as the region of Veneto (9%) and Trentino Alto Adige (14%) (Gasparini *et al.*, 1992; Rendine *et al.*, 1997). Interestingly, the parents of

the CF patients born in SC carrying R1162X were all of Italian descent. This finding is in agreement with Italian immigration to Brazil, which consisted mainly of inhabitants of the Veneto region, accounting for 48% of all Italian immigrants. In certain southern Brazilian regions, such as SC, this percentage has been as high as 90% (Hutter, 1987; Pereira *et al.*, 1999). The R1162X mutation is the second most common mutation identified to date in Afro-Brazilian CF patients (see Table 1).

G85E

With an overall frequency of 2.3%, G85E is the sixth most common mutation identified to date in the Euro-Brazilian and Afro-Brazilian CF patients, (2.6% and 1.3%, respectively). Although G85E is not included in the 10 most frequent CF mutations worldwide (CFGAC, 1994) and neither is it among the five most frequent mutations in our sample, it is interesting to note that this mutation was found in all states and both population subgroups analyzed in this study, probably as the result of admixture with Euro-Brazilians.

Two other mutations, R553X and G551D, are common worldwide, but are rare in Brazil.

3120+1G \rightarrow A

This mutation was detected in both Afro- and Euro-Brazilian CF patients. It accounts for 2.6% of Afro-Brazilians CF alleles, as opposed to 12.5% of Afro-American CP alleles (Macek *et al.*, 1997). Although we report a lower frequency of this mutation in the Afro-Brazilian CF patients, this is, as far as we know, the first description of a 3120+1G \rightarrow A mutation in Caucasian CF patients. These data suggest that the 3120+1G \rightarrow A mutation is expected to be present with a higher frequency in CF alleles in some Brazilian states, mainly the ones that have a high percentage of Afro-Brazilian descendants, such as MG. We believe that the overall frequency of this mutation will be higher in Euro-Brazilians than in U.S. Caucasians, again because population admixture is higher in Brazil (Salzano and Freire Maia, 1967). As this mutation also has been identified in four native African CF patients, on five of eight chromosomes (Carles *et al.*, 1996), consistent with its origin before the Bantu expansion roughly 2000 years ago (Carles *et al.*, 1996), the presence of mutation 3120+1G \rightarrow A in both Afro- and Euro-Brazilians is not totally surprising. Brazil imported a large number of African slaves during the 16th to the 19th centuries, and admixture with those of European descent has been high subsequently.

Mutation 3120+1G \rightarrow A appears to be an ancient mutation that may be more common than previously thought in populations of the tropical and subtropical belt, where CF is an underdiagnosed disorder. We say "underdiagnosed," because failure to thrive and diarrhea due to CF can be difficult to distinguish from other, more common causes, such as viral or parasite infection and malnutrition (Dork *et al.*, 1998). This may be the case in Brazil.

Forty-seven *CFTR* mutations were not detected in any of the 386 CF alleles studied (see Table 1). Our data show that screening for 23 of these 70 *CFTR* mutations enables sensitivity to reach 69% in Brazilian CF. In Euro-Brazilians, these 23 mutations account for 81% of the CF alleles, a detection rate that differs from the 90% reported for northern European and North

American CF subjects, yet similar to the rate (around 75%) seen in southern European countries, agreeing with the predominant colonization of Brazil by immigration from southern Europeans (Nunes *et al.*, 1991; CFGAC, 1994; Raskin *et al.*, 1999; Estivill *et al.*, 1997; Heim *et al.*, 2001). The different mutation frequencies found between individual states indicate that each state of Brazil may need a specific CF mutation database (Table 1).

We also found different mutation frequencies in CF patients from the two population subgroups studied (Afro- and Euro-Brazilians). The Euro-Brazilians were discussed previously; in the Afro-Brazilian group, only 21% of CF mutations were detected using our test panel, with only seven different mutations found. This suggests that the original panel of mutations selected for this study is not adequate for all Brazilian CF patients. Of the 70 CF mutations included in that panel, 66 are generally considered "Caucasian" mutations, and 4 are known as "common African" mutations (Macek *et al.*, 1997). Screening for the 66 "Caucasian" mutations identified only 18.5% of CF alleles in the Afro-Brazilians, while screening for the 4 "common African" mutations accounted for an additional 2.6% (total, 21.1%). This suggests that Afro-Brazilians may have a *CFTR* mutation pool that is different from that in Afro-Americans. This is supported by the fact that, although admixture is known to be higher in Brazil than in the United States, screening for 16 "Caucasian" mutations identified 52% of CF alleles in African-Americans, while screening for the same 4 "common African" mutations accounted for an additional 17% (total, 69%) (Macek *et al.*, 1997).

The incidence of CF in the Afro-Brazilian population is probably higher where considerable intermixing with Euro-Brazilians has occurred. It has already been shown that ~1/15,300 individuals of African descent living in the United States is born with CF (Hamosh *et al.*, 1998). The presence in Afro-Brazilians of at least four CF alleles that are common in Caucasians (*i.e.*, $\Delta F508$, G542X, G85E, R1162X) indicates that the incidence of CF in Afro-Brazilians is due, at least in part, to genetic admixture. However, admixture alone does not totally account for the occurrence of CF in Afro-Brazilians, as is evidenced by the fact that 79% of CF alleles in this population subgroup remain unidentified after screening for 66 common "Caucasian" *CFTR* mutations. Also, finding the most frequent African CF mutation (3120+1G \rightarrow A) in 2 CF patients of presumed European origin shows how difficult it can be to establish criteria differentiating those of European from those of African descent in the current Brazilian population.

Even Brazilians who are phenotypically Caucasian in appearance and consider themselves to be of European origin may not be completely free of African admixture. The high admixture rates in the Brazilian population may not only transfer CF alleles from Euro- to Afro-Brazilians (as with $\Delta F508$), but also vice versa, as with 3120+1G \rightarrow A. This possibility must be taken into account when designing any panel of mutations with which Brazilian CF patients are to be screened.

This work also demonstrates that in heterogeneous populations, such as the Brazilians, one must be very careful in estimating an "overall" prevalence of particular mutations (such as $\Delta F508$). For example, the frequency of $\Delta F508$ was previously reported to be 47% in Euro-Brazilians with CF (Raskin *et al.*, 1993), and a similar frequency was found for the Euro-Brazilians from the three states included in our study. However, the

overall prevalence of this mutation in all CF patients would be estimated to be lower because the Afro-Brazilian proportion of the population, in which $\Delta F508$ is much less common, is significant in all of the states of Brazil and is higher than 50% in 16 of 27 states (IBGE, 2002).

The fact of *CFTR* allelic heterogeneity has important consequences for both genetic counseling and CF population screening in the Brazilian population. Our data show that 23 mutations will pick up 80% of Euro-Brazilian *CFTR* mutations overall in the three states studied, and only 13 mutations account for 95% of the CF alleles in patients and all carrier couples in the SC (Table 1). Considering the heterogeneity of the Brazilian population in terms of racial and ethnic origins, as well as the intraregional migration flow, one might expect that the frequency of CF mutations, as well as the incidence of CF, would vary between different regions and populations of Brazil. The regional differences in Euro- and Afro-Brazilian composition will influence both cost-benefit analyses and risk assessments, and, therefore, uniform policies regarding population screening of CF patients and carriers may be difficult to establish in a country like Brazil.

Studies are in progress to estimate the incidence of CF in Brazil (Raskin *et al.*, 2003), because the disease is underdiagnosed in Brazil and throughout Latin America. Although a general population screening plan in the entire country may not be feasible, it is now possible to think about establishing a CF neonatal screening program in the Southern states of Brazil using a two-tiered approach (biochemical plus DNA test) (Raskin *et al.*, 1992a,b; Chakraborty *et al.*, 1993; Raskin *et al.*, 2003). To improve the detection rate of CF mutations in this heterogeneous population, a screening panel, specifically including "southern European Caucasian" and "African" mutations, will be needed (Heim *et al.*, 2001; Bobadilla *et al.*, 2002). The composition of such screening panels should also be enhanced by knowledge gained from complete *CFTR* gene sequencing, now available, which makes it possible to identify all mutations in CF affecteds in whom one or both mutations have been missed by current panels.

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