3. RESULTATS

Els resultats de la tesi s'han dividit en tres apartats. El primer recull cinc articles que descriuen l'heterogeneïtat molecular de la FQ a la població espanyola i la seva aplicació al diagnòstic prenatal. En el decurs dels anys en els que s'ha realitzat aquest treball la doctoranda ha participat activament en la realització dels estudis moleculars, anàlisi i interpretació de resultats, preparació i elaboració de manuscrits i organització general del treball de laboratori de diagnosi de fibrosi quística i malalties relacionades. En aquest periode varis membres del laboratori han participat en aquestes tasques, entre els que he de destacar a Javier Giménez i a Loli Ramos.

El segon apartat inclou un article amb l'estudi de la correlació genotip-fenotip com aproximació per definir la gravetat de les mutacions i el seu pronòstic clínic. D'aquest treball vull assenyalar l'aportació de dades clíniques que han fet les Unitats de FQ, tan de dintre com de fora de Catalunya.

El tercer apartat recull tres articles on s'analitzen fenotips relacionats amb FQ, un d'aquests treballs encara està pendent d'acceptació. La selecció i descripció acurada dels pacients s'ha aconseguit gràcies a la tenacitat i rigor de Lluís Bassas, Antoni Farré, Lluís Aparisi i Javier de Gracia.

Caracterització molecular de la FQ a la població espanyola

Cystic fibrosis in the basque country: high frequency of mutation ΔF508 in patients of basque origin

T. Casals, C. Vázquez, C. Lázaro, E. Girbau, FJ. Giménez, X. Estivill

American Journal of Human Genetics 1992, 50: 404-410.

Cystic fibrosis in Spain: high frequency of mutation G542X in the mediterranean coastal area

T. Casals, V. Nunes, A. Palacio, J. Giménez, A. Gaona, N. Ibáñez, N. Morral, X. Estivill

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Prenatal diagnosis of cystic fibrosis in a highly heterogeneous population

T. Casals, J. Giménez, MD. Ramos, V. Nunes, X. Estivill

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High heterogeneity for cystic fibrosis in Spanish families: 75 mutations account for 90% of chromosomes

T. Casals, MD. Ramos, J. Giménez, S. Larriba, V. Nunes, X. Estivill

Human Genetics 1997, 101: 365-370.

Paternal origin of a *de novo* novel *CFTR* mutation (L1065R) causing cystic fibrosis

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Human Mutation 1998, 1: S99-S102.

Am. J. Hum. Genet. 50:404–410, 1992

Cystic Fibrosis in the Basque Country: High Frequency of Mutation ΔF508 in Patients of Basque Origin

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Summary

The Basque population is one of the oldest populations of Europe. It has been suggested that the Basques arose from a population established in western Europe during the late Paleolithic Age. The Basque language (Euskera) is a supposedly pre-Indo-European language that originates from the first settlers of Europe. The variable distribution of the major cystic fibrosis (CF) mutation (ΔF508 deletion) in Europe, with higher frequencies of the mutation in northern Europe and lower frequencies in southern Europe, has suggested that the ΔF508 mutation was spread by early farmers migrating from the Middle East during the Neolithic period. We have studied 45 CF families from the Basque Country, where the incidence of CF is approximately 1/4,500. The birthplaces of the parents and grandparents have been traced and are distributed according to their origin as Basque or Mixed Basque. The frequency of the ΔF508 mutation in the chromosomes of Basque origin is 87%, compared with 58% in those of Mixed Basque origin. The analysis of haplotypes, both with markers closely linked to the CF gene and with intragenic markers, suggests that the ΔF508 mutation was not spread by the Indo-European invasions but was already present in Europe more than 10,000 years ago, during the Paleolithic period.

Introduction

Cystic fibrosis (CF) is the most common serious recessive disease in the caucasoid population, with an incidence ranging between 1/1,700 and 1/7,700 (Boat et al. 1989). The CF gene has been cloned, and the major mutation—a deletion of 3 bp at codon 508 (ΔF508) of the predicted protein (CF transmembrane conductance regulator [CFTR])—has been identified (Kerem et al. 1989; Riordan et al. 1989; Rommens et al. 1989).

Several studies have been reported showing the considerable variation in the distribution of the ΔF508 mutation in different populations, with frequencies of 87% in the Danish (Schwartz et al. 1990), 75%–80% in the British (McIntosh et al. 1990; Santis et al. 1990; Schwarz et al. 1990; Watson et al. 1990), 75% in North Americans (Kerem et al. 1989; Lemna et al. 1990) 50% in the Spanish (Estivill et al. 1989); 45%–55% in the Italians (Cremonesi et al. 1990; Novelli et al. 1990), 30% in the Ashkenazim (Lemna et al. 1990), and 27% in the Turks (Hundrieser et al. 1990) populations.

The lower frequencies of the mutation that are observed in the southern European populations suggest that a greater mutation heterogeneity might be present in southern Europeans compared with northern Europeans (Estivill et al. 1989). In addition, haplotype analysis with XV-2c and KM.19 markers shows a lower degree of association with the ΔF508 mutation in southern European countries than in northern European countries, suggesting that the ΔF508 mutation was introduced more recently into northern Europe than into southern Europe. On the basis of these findings it has been proposed that the ΔF508 mutation was spread in Europe by the migrations of early farmers from the Middle East, during the Neolithic period (i.e., Indo-Europeans) (EWGCFG 1990).

Received April 15, 1991; final revision received October 15, 1991.

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0002-9297/92/5002-0017$02.00

The Basque Country is situated in the western part of the Pyrenees, between France and Spain. The Basque population has several appealing features for genetic studies. The Basque population is one of the oldest populations of Europe, and it has been suggested that the Basques are derived from a population established in western Europe during the late Paleolithic period (Mourant 1983). The Basque language (or Euskera) is a pre-Indo-European language that originates from the first settlers of Europe and that has survived while other primitive languages have disappeared among successive waves of colonization by people who implanted new languages (Renfrew 1988; Mallory 1989). With regard to the polymorphic loci that have been analyzed in the Basque population, the most interesting findings are the high frequency of the Rh^- blood group and the low frequency of blood group B (Mourant et al. 1976).

We present here a study of the genetics of CF in the Basque Country, in order both to compare the isolated Basque population with the Spanish and other caucasoid populations and to test the hypothesis of a pre-Indo-European origin of the ΔF508.

Subjects and Methods

Families

Forty-five families with at least one affected CF child were studied. Diagnosis of CF was confirmed both by the typical symptoms and by two positive sweat tests. The birthplaces and the surnames of the parents and grandparents were traced and distributed according to their origin as Basque or Mixed Basque. Autochthonous individuals were distinguished according to the criteria of the eight surnames of the four grandparents being of Basque origin, each person having two surnames, the first from his or her father and the second from his or her mother. Grammatically, Basque surnames are very easy to distinguish from Catalan, French, Spanish, or other languages. The second criteria for Basque origin was that the eight great-grandparents of each CF individual were born in the Basque Country. In each individual the origin of each chromosome was ascertained independently according to these criteria.

DNA Analysis

Genomic DNA from whole blood containing EDTA as anticoagulant was extracted from the parents, the CF individuals, and, in some cases, the grandparents. DNA was subjected to amplification as recommended by the manufacturer of *Taq* polymerase. Each 50-μl reaction mixture contained 50 mM KCl, 10 mM Tris HCl pH 7.8, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 30 pM of each oligonucleotide primer, 300 ng of genomic DNA, and one unit of *Taq* polymerase.

We studied four two-allele polymorphic loci: pXV-2c/*Taq*I, pKM.19/*Scr*fI, pKM.19/*Pst*I, and pMP6d-9/*Msp*I. The loci were analyzed by restriction-enzyme digestion after PCR amplification (Saiki et al. 1988) with the primers described elsewhere (Huth et al. 1989; Rosenbloom et al. 1989; Anwar et al. 1990; Nunes et al. 1990). A CA/GT microsatellite of intron 8 of the CF gene was also analyzed in the families of Basque origin; amplification was performed using oligonucleotide primers flanking the microsatellite (Morral et al. 1991). Sequences for the amplification of the exon 10 region containing the ΔF508 mutation were obtained from Riordan et al. (1989). For the detection of the ΔF508 mutation, 5 μl of formamide-dye mixture (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol, and 20 mM EDTA) were added to 12 μl of the amplified DNA. Samples were loaded onto a 1-mm thick, 20 × 20-cm, 6% PAGE (1.6 M urea) in 1 × TBE buffer. Electrophoresis was performed at 400 V for 2 h. Fragments of either 95 bp (ΔF508) or 98 bp (normal) were observed. Analysis of the CA/GT repeat of intron 8 was as described elsewhere (Morral et al. 1991).

Results and Discussion

Only 30 of the 90 CF chromosomes are of Basque origin. The ascertainment of the origin of the chromosomes has been performed very carefully by using both the surnames and the birthplaces of the four grandparents (eight surnames) of every CF child. The high immigration that the Basque Country has suffered during this century makes it very difficult to estimate either the total number of "pure" Basque chromosomes or the incidence of the disease in the Basque population; our best estimate, 1/4,500, is based on clinical cases and neonatal screening.

The frequency of the ΔF508 mutation in the CF chromosomes of Basque origin is 86.7%, compared with 58.3% in those of Mixed Basque origin ($\chi^2 = 7.3$; $P < .007$). The frequency of the ΔF508 mutation in Basque CF chromosomes contrasts considerably with the 50% found in the overall Spanish population ($\chi^2 = 15.0$; $P < .0001$) (Chillón et al. 1990) (table 1). The higher (58.3%) frequency of the ΔF508 mutation in Mixed Basque chromosomes, compared with the

Table 1

Distribution of ΔF508 and Non-ΔF508 CF Chromosomes in Basque Population

	No. (frequency) IN		
MUTATION	Basque[a]	Mixed Basque[b]	Spanish[c]
ΔF508.....	26 (.87)	35 (.58)	194 (.50)
Non-ΔF508.....	4 (.13)	25 (.42)	194 (.50)
Total	30	60	388

[a] SE = .062.
[b] SE = .064.
[c] Source: Chillon et al. 1990. SE = .025.

50% in the Spanish chromosomes, although not significant (χ^2 1.4; P = .23), is probably due to the fact that approximately half of the Mixed Basque CF chromosomes are from regions close to the Basque Country, in which higher frequencies of the mutation have been found (T. Casals and X. Estivill, unpublished data). Although significant, the high frequency of the ΔF508 mutation found in the Basque CF chromosomes could be due to genetic drift, to a founder effect, or simply to the small size of the sample of chromosomes analyzed (table 1).

Data to support an ancient origin of the ΔF508 mutation in the Basque population could be obtained by analyzing haplotypes with markers closely linked to the gene and intragenic markers. Table 2 shows the haplotype distribution of CF and normal chromosomes according to their origin. The CF mutation is mainly associated with haplotype *1 1 2 2* (73%), which is present in only 10% of normal chromosomes of Basque origin. Data obtained for the general Spanish population show that 94% of ΔF508 chromosomes are of haplotype *1 1 2 2* (Casals et al., in press). It is interesting to note that only 68% (21 of 31) of the Mixed Basque ΔF508 chromosomes are of haplotype *1 1 2 2* (74% haplotype *B*, XV-2c/KM.19), whereas data for Spanish chromosomes in general show frequencies of 85%–94% for haplotype *B* (Chillón et al. 1990; EWGCFG 1990; Casals et al., in press). We do not have a satisfactory explanation for this discrepancy in the data, but it could reflect the fact that a high proportion of these Mixed Basque chromosomes are from regions near the Basque Country. It seems that in the past the geographical region occupied by the Basques—or by a population closely related to them—was not limited to what is known today as the Basque Country but extended into the nearby regions. Thus, some Mixed Basque chromosomes could also reflect the ancient origin of the ΔF508 mutation.

If we assume that the original haplotype in which the ΔF508 mutation arose was haplotype *1 1 2 2*, then

Table 2

Haplotypes of CF and Normal Chromosomes for DNA Markers Closely Linked to CF in Basque Population

	No. IN					
	Basque			Mixed Basque		
HAPLOTYPE[a]			Normal			Normal
T S P M	ΔF508	Non-ΔF508	Chromosomes	ΔF508	Non-ΔF508	Chromosomes
1 1 2 2....	19	...	3	21	5	6
1 2 2 2....	1	...	1	1	2	2
2 2 2 2....	3	1	4	1	4	7
2 1 2 2....	3	...	...	6	...	1
2 2 1 1....	...	2	10	...	3	19
2 1 1 1....	...	...	1	...	...	2
1 2 1 1....	...	1	8	...	3	9
1 1 1 1....	...	...	...	...	1	2
2 2 1 2....	...	...	...	...	2	...
1 2 1 2....	...	...	1	1	1	1
2 2 2 1....	...	...	2	...	...	1
1 1 2 1....	...	...	...	1	...	...
Phase unclear..	...	...	...	4	4	10
Total.....	26	4	30	35	25	60

[a] T = XV-2c/*Taq*I; S = KM.19/*Scr*fI; P = KM.19/*Pst*I; and M = MP6d-9/*Msp*I.

Table 3

Haplotype Percentage for XV-2c and KM.19 Polymorphisms in ΔF508 CF Chromosomes in Seven European Populations

	% IN[b]						
HAPLOTYPE[a]	Danish	Dutch	Belgian	British	Italian	Spanish	Basque
A	.4	1.8	.7	.9	3.4	3.7	1.8
B.....	97.3	96.9	97.9	94.9	87.9	84.9	75.4
C	.4	. . .	. . .	1.5	2.5	2.7	. . .
D	1.9	1.3	1.4	4.0	6.2	8.7	22.8

[a] Letters are those assigned by Estivill et al. (1987).

[b] Data on Danish, Dutch, Belgian, British, Italian, and Spanish populations are from EWGCFG 1990; Basque chromosomes are the total in the Basque Country, independently of their origin (57 chromosomes; table 2).

several recombinational phenomena in the ΔF508 chromosomes have occurred both between the pXV-2c/*Taq*I and pKM.19/*Scr*fI loci and between the pKM.19/*Scr*fI and pXV-2c/*Taq*I loci. However, a similar number of recombinational events are also detected in the Mixed Basque ΔF508 chromosomes, in which two haplotypes—*1 1 2 1* and *1 2 1 2*—probably originated by recombinations between pKM.19/*Pst*I and pMP6d-9/*Msp*I and between the latter and the ΔF508 mutation. The recombinations in the Basque and in some Mixed Basque chromosomes suggest that the ΔF508 mutation was not introduced into the Basque Country recently. If, for pXV-2c/*Taq*I and pKM.19/*Pst*I, we compare the data obtained from Danish, Dutch, Belgian, British, Italian, and Spanish chromosomes with the data from the Basque Country chromosomes, there is a gradient in the frequency of haplotype *B* (EWGCFG 1990; also see table 3). The highest (about 97%) frequency of haplotype *B* associated with mutation ΔF508 is found in the Danish, Dutch, Belgian, and British populations, and frequencies of 88% and 85% are found in the Italian and Spanish populations, respectively, in contrast with the 75% frequency found in the Basque population (table 3).

It is interesting to note that in some eastern European countries (Finland, Poland, and the USSR), in which the frequency of the ΔF508 mutation is approximately 50%, the ΔF508 mutation is almost exclusively associated with haplotype *B* (EWGCFG 1990; Kere et al. 1990; Bal et al. 1991). Thus, data on haplotypes and frequency of the ΔF508 mutation suggest that the mutation has been introduced more recently into both northern and eastern Europe than into southern Europe (table 3).

Additional information on the origin of ΔF508 chromosomes is obtained with the analysis of the intron 8 microsatellite (Morral et al. 1991). Three microsatellite alleles were found associated with the ΔF508 mutation in the patients of Basque origin—61.5% with allele *2*, 30.8% with allele *6*, and 7.7% with allele *7*. Alleles *6* and *7* differ only by one CA/GT dinucleotide, but alleles *2* and *6* differ by six dinucleotides. Although in the Spanish chromosomes the ΔF508 mutation is associated with five different alleles, when the complete haplotypes, which include the five markers, are considered, there is more variation in the Basque chromosomes than in the Spanish chromosomes (table 4). Thus, the diversity index—$I = 1/(\Sigma P_i^2)$—is higher in the Basques than in the Spanish (3.13 vs. 2.52). This variation in haplotypes in the Basque chromosomes may be related to the mutation having been present for a longer period in the Basque population than in the general Spanish population (Morral et al. 1991). Haplotype data (including data on the intron 8 microsatellite) for other European populations should provide relevant information on the origin and diffusion of the ΔF508 mutation.

The Basque population has remained isolated for centuries. Data on the history, language, and culture of the Basque population indicate that the Basques remained untouched by the eastern European invasions of the Iron Age. Genetic, linguistic, and archaeological studies suggest that the Basques had scarce contacts with the Celts and Iberians (Ammerman and Cavalli-Sforza 1984; Piazza et al. 1981, 1988). However, data on the Iberians are controversial, as the Basque language and Iberian language could have the same origin. During the Roman Empire, only the areas near the borders of the Basque Country were of inter-

Table 4

Haplotype Distribution for Polymorphic Loci 5′ to CFTR Gene and Intron 8 Microsatellite in Basque and Spanish Chromosomes

Haplotype[a]					No. in			
					Basque		Spanish	
T	S	P	M	CA/GT (N^c)	ΔF508	Non-ΔF508	ΔF508	Non-ΔF508
1	1	2	2	2 (23)	13	. . .	42	33
1	1	2	2	6 (17)	6	. . .	35	. . .
1	1	2	2	3 (22)	. . .	. . .	2	2
1	1	2	2	1 (24)	. . .	. . .	1	. . .
1	1	2	2	7 (16)	. . .	. . .	2	4
1	2	2	2	6 (17)	1	. . .	. . .	3
2	2	2	2	2 (23)	1	. . .	. . .	. . .
2	2	2	2	7 (16)	2	1	. . .	3
2	1	2	2	2 (23)	2	. . .	2	. . .
2	1	2	2	7 (16)	. . .	. . .	2	. . .
2	1	2	2	6 (17)	1	. . .	. . .	. . .
1	2	1	2	7 (16)	. . .	. . .	1	. . .
Subtotal.....					26	1	87	45
Others.....					. . .	3	. . .	57
Total					26	4	87	102

[a] Letters are as in table 2.
[b] Source: Morral et al. (1991).
[c] Number of dinucleotide repeats.

est to the Romans, so the interior of the country remained intact. Thus, the autochthonous component of the Basque population genetic structure did not receive any new genetic contributions until the beginning of the present century, when the Basque Country suffered a relevant influx of migrants from the center and south of Spain.

The Basque language (Euskera) is a pre-Indo-European language. Recent hypotheses on the ancestral Indo-European language suggest that it was introduced by farmers from Anatolia who moved in waves through Europe 5,000–10,000 years ago (Renfrew 1988). It is believed that the Basque language has the oldest origin of all spoken languages in Europe.

The differences between the Basques and other populations are seen not only in the Basque language and culture but also in the frequency of genetic markers. Several polymorphic loci have been analyzed in the Basque population (Pancorbo et al. 1983, 1986, 1989; Aguirre et al. 1989). The heterogeneity between the Basques and neighboring populations underlies the genetic isolation of the Basques. However, the most striking differences identified are the high frequency of the Rh^- blood group (the Basques have the highest Rh^- frequency in the world) and the low frequency of blood group B (the lowest in Europe) (Goti-Iturriaga 1966; Mourant et al. 1976). These data suggest that, of any extant group, the Basque population comes closest to being remnants of the original European population.

The data presented here—i.e., the high frequency of the ΔF508 mutation and the haplotype variation in the Basque population—together with the previously reported high frequency of this mutation in northern European countries suggest that the ΔF508 mutation was already present in Europe before the Indo-European migrations, which occurred 5,000–10,000 years ago. The findings of the present study do not refute the hypothesis that most modern European people are descendants of migrations from the Middle East during the Neolithic period (Cavalli-Sforza 1988; Renfrew 1988). However, the hypothesis of a recent report (EWGCFG 1990), suggesting that the diffusion of the ΔF508 mutation took place during the Neolithic period and that the spread of the mutation accompanied the migrations of early farmers from the Middle East toward the northwest of Europe, has several contradictory points: (1) Higher frequencies of the mutation among the CF chromosomes overall should be expected where these migrants were established for a longer period (i.e., in eastern and southern Europe).

(2) If mutation ΔF508 accompanied the Neolithic migrations, then a selective effect against the mutation should be postulated in the regions where the mutation entered first, favoring also its prevalence where it was introduced later (selective forces for the mutation would appear in some regions and would disappear in others). (3) Haplotypes on ΔF508 chromosomes should accompany the diffusion of the ΔF508 mutation, with a wide haplotype diversity where the mutation was introduced earlier.

Our findings in the Basque population suggest that the ΔF508 mutation is probably a very old molecular defect. This mutation would have been present in Europe in a Paleolithic population of which the Basques are the most homogeneous relic population. Data on the diffusion of agriculture, the linguistic transformation of Europe, and the frequency of the ΔF508 mutation in different European countries suggest also that the Neolithic migrations diluted the frequency of the ΔF508 mutation in some populations, by bringing other CF mutations into Europe. These mutations would be more frequent in the regions in which the Indo-Europeans were better established (mainly where climate was more appropriate for agriculture), keeping the frequency of the ΔF508 mutation lower than that where they had either fewer or more recent contacts, as has occurred in the Basque Country.

Further confirmation of the hypothesis presented here of a Paleolithic origin of the ΔF508 mutation should be achieved when a larger Basque sample is analyzed. However, the study of microsatellite markers in other European populations should provide further proof for this hypothesis. On the other hand, the frequency and geographical distribution of other common CF mutations might reveal relevant information on the mutations that accompany the Neolithic migrations.

Acknowledgments

We thank Virginia Nunes and Núria Morral for useful comments on the manuscript, and thank the "Fondo Investigaciones Sanitarias de la Seguridad Social" (grant 90E1254) and Institut Català de la Salut.

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human genetics

Cystic fibrosis in Spain: high frequency of mutation G542X in the Mediterranean coastal area

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Received: 10 April 1992 / Revised: 22 July 1992

Abstract. We have determined the frequency of deletion ΔF508 and mutation G542X, a nonsense mutation in exon 11 of the cystic fibrosis (CF) gene, in a sample of 400 Spanish CF families. Mutation G542X represents 8% of the total number of CF mutations in Spain, making it the second most common mutation after the ΔF508 deletion, which accounts for 48% of CF chromosomes. G542X has a higher frequency in the Mediterranean coastal area (14%) and in the Canary Islands (25%). About 70% of G542X chromosomes are from Andalucia, Múrcia, Valencia, Catalunya and the Canary Islands. The ΔF508 deletion has its highest frequency in the Basque Country (83%). Mutation G542X is associated with the same rare haplotype that is found in association with the ΔF508 mutation. The haplotype homogeneity found for G542X, even when intragenic microsatellites (IVS8CA, IVS17BTA and IVS17BCA) are considered, allows us to postulate that this mutation arose from a single mutational event. The geographic distribution of mutations ΔF508 and G542X suggests that ΔF508 was present in the Iberian Peninsula before the Indo-European invasions, and that G542X was introduced into Spain, via the Mediterranean Sea, probably by the Phoenicians, between 2500 and 3000 years ago.

Introduction

Cystic fibrosis (CF) is believed to be the most common severe autosomal recessive disease in the Caucasoid population, with an average incidence of approximately 1 in 2000 individuals (Boat et al. 1989). The CF transmembrane conductance regulator (CFTR) gene has been identified as the gene mutated in CF (Rommens et al. 1989; Riordan et al. 1989). The major CF mutation (ΔF508 deletion) (Kerem et al. 1989) accounts for about 70% of North American and North European CF chromosomes, but is present in only 50% of CF chromosomes from the Mediterranean region (EWGCFG 1990; Cystic Fibrosis Genetic Analysis Consortium 1990).

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Molecular analysis of the CFTR gene has lead to the identification of more than 170 mutations (Cystic Fibrosis Genetic Analysis Consortium, unpublished data). However, most of the identified mutations (excluding ΔF508) are uncommon in the total patient population, and only a few of them have a discrete frequency in specific populations (Cutting et al. 1990; Kerem et al. 1990; Cuppens et al. 1990; Rozen et al. 1992; Nunes et al. 1991; Gasparini et al. 1992).

In our analysis of 15 CF mutations in patients from the Mediterranean area (Nunes et al. 1991), we have found that the second most common CF mutation is G542X, a nonsense mutation in exon 11 of the CFTR gene (Kerem et al. 1990). This mutation has a relatively high frequency in Spain, accounting for approximately 8% of CF chromosomes. We present here an extended analysis of the prevalence of mutations G542X and ΔF508 in the Spanish population, their geographic distribution, and the association of mutation G542X with haplotypes of closely linked, intragenic microsatellite markers.

Materials and methods

Patients and families

Some of the Spanish CF families analyzed here have been reported elsewere (Chillón et al. 1990; Casals et al. 1991). In total, 400 CF families, 377 with at least one affected CF child and 23 in which the CF child was deceased, have been analyzed. CF diagnosis was based on the characteristic clinical features of the disease and at least two positive sweat tests; for the deceased children, diagnosis was based on postmortem data and mutation analysis of the parents.

The geographic origin of the CF chromosomes was ascertained by analyzing the birthplace of parents and the four grandparents. For the geographic distribution of the mutations, only unambiguous data was analyzed, and only chromosomes of Spanish origin were considered in the analysis.

Mutation and haplotype polymorphism analysis

Standard methods were used for polymerase chain reaction (PCR) analysis of the restriction fragment length polymorphisms (RFLPs) D7S23 and D7S399, which are closely linked to the CF locus, and for the ΔF508 mutation (Casals et al. 1991). The G542X mutation

was detected via two different methods: (1) hybridization with allele-specific oligonucleotide (ASO) probes [normal: 5'ACCTTCTCCAAGAACT, and G542X: 5'ACCTTCTCAAAGAACT (Kerem et al. 1990)], exon 11 being amplified by primers 11i-5: 5'CAACTGTGGTTAAAGCAAATAGTGT and 11i-3: 5'GCACAGATTCTGAGTAACCATAAT (Zielenski et al. 1991a); (2) by PCR amplification with a modified primer that creates a restriction site near the mutated nucleotide (PCR-mediated site-directed mutagenesis) (Haliassos et al. 1989) [normal: 5'CTTGCTAAAGAAATTCTTGG, and the modified primer: 5'CAGAGAAAGACAATATAGTTCCT, in which the penultimate base T has been substituted for a C, creating a restriction site for *Scr*FI, which is abolished in the G to T G542X mutation (Gasparini et al. 1992)]. Amplification of three microsatellites, IVS8CA (intron 8), and IVS17BTA and IVS17BCA (intron 17B), were as described (Estivill et al. 1991; Morral et al. 1991, 1992; Zielenski et al. 1991b).

Results and discussion

G542X and ΔF508 frequencies and genotypes

We have studied 400 Spanish CF families for mutations G542X and ΔF508. In total, 420 CF patients, 800 parents and 414 sibs and other relatives were analyzed (1634 DNA samples). The proportion of ΔF508 is 48.1% (95% confidence interval 44.6%–51.6%), a similar figure to the 50% reported previously (Estivill et al. 1989; Chillón et al. 1990). G542X accounts for 8.1% (95% confidence interval 6.2%–10%) of the total number of CF chromosomes, being the second most common mutation in Spain. The group of "other" mutations, which include known (about 8%) and as yet uncharacterized (35%) mutations, accounts for 43.8% of CF chromosomes (95% confidence interval 40.4%–47.2%) (Nunes et al. 1991).

The genotype distribution of the ΔF508 and G542X mutations and the "other" mutations is shown in Table 1. The genotype distribution does not follow the Hardy Weinberg equilibrium (Li 1976) ($X_1^2 = 20.77$; $P < 0.001$), which suggests that the population analyzed is not homogeneous for the distribution of these two mutations and the "other" mutations group.

Table 1. Distribution of CF genotypes in 400 Spanish CF-patients[a]

Genotype	Observed *n* (%)	Expected *n* (%)
ΔF508/ΔF508	113 (28.25)	93 (23.2)
ΔF508/G542X	30 (7.5)	31 (7.8)
G542X/G542X	5 (1.25)	3 (0.7)
ΔF508/Other	129 (32.25)	168 (42.1)
G542X/Other	25 (6.25)	28 (7.1)
Other/Other	98 (24.5)	77 (19.1)
Total	400 (100.00)	

[a] Only one affected individual from each family has been considered

Geographic distribution of G542X and ΔF508

It was possible to trace the geographic origin of mutations ΔF508, G542X, and the unknown "other" mutations for 600 chromosomes (Table 2, Figs. 1, 2). The origin of the CF chromosomes was decided by the birth places of the four grandparents of every CF child. There was no data available from three regions: Asturias, Rioja, and the Balearic Islands.

The highest frequency of ΔF508 was found in the Basque Country (83.3%), followed by Castilla/Madrid, Valencia, and Cantabria (66.7%, 62.2% and 60.0%, respectively). The lowest frequency of ΔF508 was found in Navarra, Galicia, Múrcia and Aragón (33.3%, 36.3%, 41.1% and 41.5%, respectively) (Fig. 1, Table 2).

About 70% of G542X chromosomes are from the southeast of the country and the Canary Islands. The highest frequency of mutation G542X was found in the Canary Islands, Múrcia, Navarra, and Valencia (25.0%, 20.7%, 16.7%, and 10.8%, respectively). When the country is divided into two parts, provinces on the Mediterranean coast (i.e., (from north to south) Girona, Barcelona, Tar-

Table 2. Geographic distribution of mutations G542X and ΔF508, and unknown "other" mutations in the Spanish population

Autonomous community	Total chromosomes	Mutations		
		ΔF508 *n* (%)	G542X *n* (%)	Other *n* (%)
Andalucia	187	100 (53.4)	17 (9.0)	70 (37.4)
Aragon	65	27 (41.5)	3 (4.6)	35 (53.8)
Pais Vasco	30	25 (83.3)	0 (0.0)	5 (16.7)
Canarias	28	15 (53.6)	7 (25.0)	6 (21.4)
Cantabria	10	6 (60.0)	1 (10.0)	3 (30.0)
Castilla-Leon	61	33 (54.0)	4 (6.5)	24 (39.3)
Catalunya	42	25 (59.5)	4 (9.5)	13 (31.0)
Extremadura	23	11 (47.8)	1 (4.4)	11 (47.8)
Galicia	55	20 (36.3)	3 (5.4)	32 (58.2)
Madrid/Cast-Mancha	21	14 (66.7)	2 (9.5)	5 (37.9)
Murcia	29	12 (41.1)	6 (20.7)	11 (37.9)
Navarra	12	4 (33.3)	2 (16.7)	6 (50.0)
Valencia	37	23 (62.2)	4 (10.8)	10 (27.0)
Total	600	315 (52.5)	54 (9.0)	231 (38.5)

n, number of chromosomes

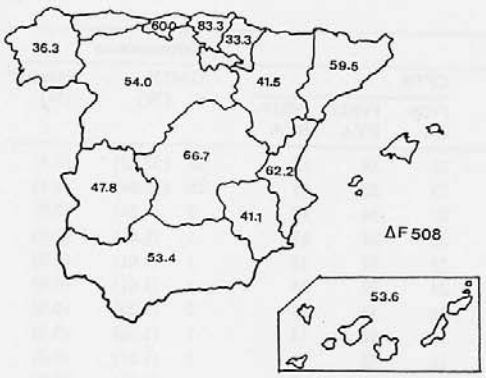

Fig. 1. Geographic distribution of mutation ΔF508 in Spain. *Numbers* represent the percentages of the mutations in the different autonomous communities of the country. Names of the communities are in Table 2.

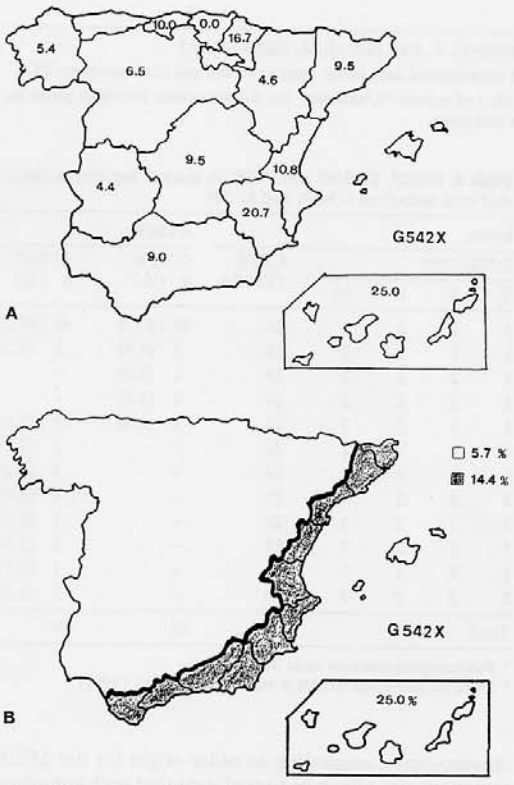

Fig. 2A, B. Geographic distribution of mutation G542X in Spain. **A** Distribution by communities; **B** distribution of G542X dividing the country into two parts: the provinces with contact with the Mediterranean Sea, and the rest of the country. *Numbers* represent percentages of the mutation

ragona, Castellón, Valencia, Alicante, Múrcia, Almería, Granada, Málaga and Cádiz) and the rest of country, 24 out of the 167 Mediterranean coast CF chromosomes (14.4%) have mutation G542X, compared with 23 out of 405 CF chromosomes (5.7%) from the rest of the country (excluding the Canary Islands) ($X_1^2 = 11.91$; $P < 0.001$; Yule's coefficient of association, $Q = 0.47$; $-1 \leq Q \leq 1$) (Fig. 2).

Origin of mutation G542X

Mutation G542X has also been found in other Mediterranean populations: Italian (4%), Greek (5%) (Nunes et al. 1991), and the Jewish population of Israel (between 8.4% and 13.5%) (Kerem et al. 1990; Lerer et al. 1991). In addition, G542X has also been found to be frequent in some Belgian patients (7.3%) (Cuppens et al. 1990), which could reflect a founder effect arising from immigration from southern Europe.

From the high incidence of mutation G542X in Spain, compared with other Mediterranean and European countries, one could hypothesize that the mutation has an Arab origin (most of Spain was dominated by the Arabs for at least seven centuries). However, the concentration of the G542X mutation on the Mediterranean coast does not coincide with the areas occupied by the Arabs in Spain.

The Iberian Peninsula was colonized by other peoples before the Arab invasions: the Neolithic/Indo-Europeans, the Phoenicians, the Greeks, and the Romans. The Phoenicians lived in the eastern Mediterranean area, regions that are now Syria, Lebanon, and north Israel. After the eighth century B.C., the Phoenicians increased their commercial activity in the Mediterranean; they exploited the silver mines of southern Spain for about two centuries. In the sixth century B.C., the Phoenicians initiated a new wave of colonization of the Iberian Peninsula, leading to the establishment of colonies in the south and east of Spain (Harrison 1988; Petit 1962).

The Ashkenazic Jews have a high frequency of mutation G542X (13.5%) Lerer et al. 1991), but not the Sephardic Jews, among whom G542X is uncommon (Shoshani et al. 1991); however, the distribution of mutation G542X in Spain does not coincide with the main Jewish centers in the Iberian Peninsula. If we assume that G542X arose from a single mutational event, the high proportion of G542X found in the Jewish population could be explained as a founder effect and inbreeding. There is a link between these populations as the Hebrew and Phoenician languages belong (together with Canaanitie and Ugaritic) to the Canaanitic branch of the Semitic subfamily of the Afro-Asiatic family of languages. The Canaanites are known to have been established, about 2000 B.C., in regions that are now Syria and Palestine. The Canaanites were progressively substituted by other peoples including the Phoenicians and the Hebrews (Harrison 1988).

G542X haplotype association

The G542X mutation occurs in the same (D7S23/D7S399) haplotype in which the ΔF508 arose (Table 3), a rare

Table 3. Extragenic and intragenic DNA marker haplotypes associated with mutation G542X

Locus							Chromosomes	
D7S23/399				CFTR			G542X	Normal[a]
T	S	P	M	IVS8-CA	IVS17-BTA	IVS17-BCA	*n* (%)	(%)
1	1	2	2	23	33	13	33 (53.23)	(0.4)
1	1	2	2	23	32	13	18 (29.04)	(0.4)
1	1	2	2	23	34	13	3 (4.84)	(0.0)
1	1	2	2	23	31	13	1 (1.61)	(2.0)
1	1	2	2	23	37	13	1 (1.61)	(0.0)
1	2	2	2	23	33	13	1 (1.61)	(0.0)
1	1	2	2	16	33	13	2 (3.23)	(0.0)
1	1	2	2	16	32	13	1 (1.61)	(0.0)
1	2	2	2	16	32	13	1 (1.61)	(0.0)
1	1	2	2	17	33	13	1 (1.61)	(0.0)
Subtotal							62 (100.00)	(2.8)
Unknown				23	33	13	1	
Unknown				23	32	13	1	
Unknown				23	34	13	1	
Subtotal							3	
Total							65	

T, XV-2c/*Taq*I; S, KM.19/*Scr*fI; P, KM.19/*Pst*I; M, MP6d-9/*Msp*I

[a] Morral et al. 1991, and unpublished data (total number of normal chromosomes: 252)

CFTR alleles are the number of repeats "Unknown" are chromosomes for which phase for D7S23 and D7S399 were unknown

haplotype (less than 3%) in the current European population (Casals et al. 1991). The majority of G542X chromosomes (33 out of 62; 53.2%) are associated with the same haplotype *1 1 2 2* (D7S23/399), *23* (IVS8CA), *33* (IVS17BTA), *13* (IVS17BCA) (Table 3). As the extragenic markers and the mutation flank the IVS8CA locus, the *16* and *17* CA repeat IVS8CA alleles, associated with G542X, might have arisen from the original *23* CA repeat allele by slipped-strand misparing, as has been demonstrated for the ΔF508 mutation (Morral et al. 1991).

It is probable that the five IVS17BTA alleles associated with G542X, of which the commonest is allele *33* (59%), arose by the same mechanism described for the IVS8CA microsatellite. All G542X chromosomes are associated with the same IVS17BCA *13* allele, which is present in 68% of normal chromosomes (Morral et al. 1992). Haplotype data support the hypothesis of a single origin for the G542X mutation in the Spanish population.

A lower variability of alleles is found for the G542X mutation compared with that of ΔF508 (Table 4). Thus, the diversity index $[I = 1/(\Sigma p^2_i)]$ is higher in ΔF508 than in the G542X chromosomes (2.63 versus 1.22). As a result of this variation in haplotypes in the ΔF508 chromosomes, it could be postulated that the ΔF508 mutation is older than the G542X defect.

Based on the high frequency of the ΔF508 mutation in the Basque population, and on the heterogeneous haplotype distribution, we have hypothesized that mutation ΔF508 probably arose during the Paleolithic Age, and that the mutation was present in Europe before the Neolithic invasions (Casals et al. 1992). The different variability of alleles associated with G542X and ΔF508 chromosomes, suggesting an older origin for the ΔF508 mutation, also allows us to postulate that both mutations originated in the Caucasoid population during two distinct periods: ΔF508 during the Paleolithic Age, and G542X probably during or after the Neolithic period, in a population of Semitic origin, of which both Phoenicians and Jews are descendants.

Table 4. D7S23, D7S399 and IVS8CA marker haplotypes associated with mutations G542X and ΔF508

Locus					Mutation	
D7S23/399[a]				CFTR IVS8CA	G542X	ΔF508[b]
T	S	P	M		*n* (%)	*n* (%)
1	1	2	2	23	56 (90.3)	42 (47.2)
1	1	2	2	16	3 (4.8)	2 (2.2)
1	2	2	2	23	1 (1.6)	–
1	2	2	2	16	1 (1.6)	–
1	1	2	2	17	1 (1.6)	35 (39.3)
1	1	2	2	22	–	2 (2.2)
1	1	2	2	24	–	2 (1.1)
2	2	2	2	23	–	1 (1.1)
2	1	2	2	23	–	1 (2.2)
2	1	2	2	17	–	1 (2.2)
1	2	1	2	16	–	1 (1.1)
2	2	2	2	16	–	1 (1.1)
Total					62	89

[a] Polymorphic markers as in Table 3

[b] Data for mutation ΔF508 is from Morral et al. (1991)

The data reported here support our previous hypothesis that the ΔF508 mutation was present in Spain before the Indo-European invasions (Casals et al. 1992). The relatively low frequency of mutation ΔF508 in Aragon (41.5%) and Navarra (33.3%) might reflect that fact that these regions were the route through which the Indo-Europeans entered the Iberian Peninsula, thereby introducing other CF mutations that diluted the frequency of the ΔF508 deletion in these regions. The high proportion of G542X on the Spanish Mediterranean coast suggests that the mutation was introduced via the Mediterranean Sea; this is also supported by the low proportion of G542X among CF chromosomes in the Aragon region.

The geographic distribution of the mutation in the Iberian peninsula and the haplotype data support the hypothesis of a post-Neolithic introduction of G542X in Spain, via the Mediterranean Sea, probably by the Phoenicians, between 2500 and 3000 years ago. Haplotype data for G542X in other European populations should provide further insights about the genuine origin of this mutation.

Acknowledgements. We thank N. Cobos, S. Liñán, C. Vázquez, J. Ferrer-Calvete, J. Pérez-Frias, G. Antiñolo, A. Zurita, L. Ortigosa, H. Armas, A. Pizarro, H. Castro, T. Martínez, Luíz Ros, A. Lázaro, B. Rodríguez, M. Bosque, L. Séculi, G. Glover, J. Peña, and M. Milà, who have sent information on their CF families for analysis, and the Spanish CF families for their support. We thank H. Kruyer for comments and help with the manuscript, and V. Volpini for help with the statistics. This work was supported by the Fondo de Investigaciones Sanitarias de la Seguridad Social (90E1254) and the Institut Català de la Salut (Spain).

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PRENATAL DIAGNOSIS, VOL. 16: 215–222 (1996)

PRENATAL DIAGNOSIS OF CYSTIC FIBROSIS IN A HIGHLY HETEROGENEOUS POPULATION

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Received 24 July 1995
Revised 29 September 1995
Accepted 28 October 1995

SUMMARY

Cystic fibrosis (CF) is the most common autosomal recessive disease in Caucasian populations. The Spanish CF population is highly heterogeneous, with more than 70 different mutations causing CF. Since the *CFTR* gene was cloned, we have performed 81 prenatal diagnoses for 74 couples. Sixty-nine cases had a high risk (1/4) for CF and 12 presented a lower risk (1/240). Direct analysis was possible in 36 cases (44·4 per cent); it was necessary to combine mutation analysis with polymorphic markers in 24 cases (29·6 per cent); mutation analysis and microvillar enzymatic (MVE) analysis were combined in five cases (6·1 per cent); and in 16 cases (19·8 per cent), only indirect analysis was possible. Nine different mutations were detected in this series of families: 621+1G→T, ΔF508, 1609delCA, G542X, G551D, 1949del84, R1162X, W1282X, and N1303K. Another ten mutations were identified in these samples after prenatal diagnosis (1811+1.6kbA→G, 711+1G→T, 2869insG, G85E, 2176insC, ΔI507, 3272-26A→G, Q890X, R1066C, and 4005+1G→A). Our current strategy for molecular diagnosis of CF in the Spanish population is based, as a first step, on direct analysis for the two most frequent mutations (ΔF508 and G542X) and indirect analysis using the intragenic markers IVS8CA, IVS17BTA, and IVS17BCA. The second step consists of screening for the mutations already associated with the *CFTR* microsatellite haplotypes. The third step is a specific search for unknown mutations. While actual diagnostic methods are not automatic and robust enough for heterogeneous populations, the diagnostic strategy outlined provides rapid, accurate, and reliable prenatal diagnosis for the majority of couples.

KEY WORDS: cystic fibrosis; *CFTR*; CF; mutation analysis; heterogeneity

INTRODUCTION

Cystic fibrosis (CF) is a multisystemic disorder that predominantly affects the lung, intestine, sweat duct, and the vas deferens, but which has a highly variable clinical severity (Boat *et al.*, 1989). CF is the most common autosomal recessive disease and affects about 1 in 2500 newborns in Caucasian populations (Boat *et al.*, 1989). The gene responsible for CF, the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, was cloned in 1989 (Rommens *et al.*, 1989) and the most common mutation, ΔF508, was identified (Kerem *et al.*, 1989). Since then, more than 500 different CF mutations have been identified (Tsui, 1992; The Cystic Fibrosis Genetic Analysis Consortium, CFGAC, 1994; and personal communications).

Studies on different populations have shown considerable differences in the frequencies of the most common CF mutations (ΔF508, G542X, and N1303K) (CFGAC, 1994). Whereas mutation ΔF508 has been detected on between 30 and 90 per cent of CF chromosomes, depending on the ethnic origin of the patients (Tsui, 1992; CFGAC, 1994), there are mutations that are found almost exclusively in particular geographical regions (CFGAC, 1994). In general, heterogeneity for CF mutations increases in the north–south and west–east direction, along with a decrease in the frequency of the ΔF508 mutation. The Mediterranean area has the highest *CFTR* heterogeneity, making genetic diagnosis by mutation analysis difficult (Nunes *et al.*, 1991; Claustres *et al.*, 1993; Chillón *et al.*, 1994a,b; Chevalier-Porst *et al.*, 1994; Savov *et al.*, 1994; Bonizzato *et al.*, 1995; Kanavakis *et al.*, 1995).

CCC 0197–3851/96/030215–08

The Spanish CF population is amongst those of the Mediterranean area that shows the highest heterogeneity. We have identified 73 different mutations which represent 87 per cent of CF chromosomes (Chillón *et al.*, 1994a; Casals *et al.*, unpublished) in a sample of 650 Spanish CF families. Only ten of these mutations have a frequency higher than 1 per cent, accounting in total for 71 per cent of the CF chromosomes. Thus, molecular diagnosis of CF by the detection of the specific mutation in each patient is especially difficult in this population.

Prenatal diagnosis for CF was initiated in the early 1980s, first by enzymatic analysis in amniotic fluid (Brock, 1983; Boué *et al.*, 1986), later by indirect analysis using restriction fragment length polymorphisms (RFLPs) located near the *CF* gene (Farrall *et al.*, 1987; Strain *et al.*, 1988; Beaudet *et al.*, 1989; Casals *et al.*, 1991), and, finally, after the cloning of the *CFTR* gene, by direct analysis of mutations in the gene (McIntosh *et al.*, 1989; Novelli *et al.*, 1990; Baranov *et al.*, 1992). Direct analysis of the molecular defect represents the ideal approach for a disorder with a known gene. This is feasible for most couples in those populations with a few predominant mutations. However, this does not apply for a large number of couples (about 30 per cent in Spanish families) in populations with a high mutation heterogeneity. In these cases, a combination of direct and indirect analysis should be applied (Casals *et al.*, 1991; Estivill *et al.*, 1991). We present here our experience in 81 prenatal diagnoses performed by direct and indirect approaches in 74 couples from 650 Spanish CF families.

PATIENTS AND METHODS

All CF patients had at least two positive sweat tests (Cl^- >60 mEq/l). Since 1990 we have performed 81 prenatal diagnoses. The gestational age ranged from 10 to 20 weeks. One pregnancy involved twins and in another three, the couples were consanguineous. In six couples two independent prenatal diagnoses were performed. Sixty-nine couples had a high risk (1/4) for CF and 12 couples presented a lower risk (1/240). In 15 cases (18·5 per cent), the CF patient was deceased before the genetic study. Samples analysed for prenatal diagnoses were chorionic villus sample (CVS) in 53 cases, amniotic fluid (AF) in 26, and both CVS and AF in two cases.

DNA extraction was performed by standard methods (Kunkel *et al.*, 1977; Miller *et al.*, 1988). The following markers were used for indirect genetic analysis: metH/*Taq*I, E6/*Taq*I, PT-3/*Ban*II, XV-2c/*Taq*I, KM.19/*Pst*I, KM.19/*Scr*FI, MP6d-9/*Msp*I, G-2/*Scr*FI, and J3.11/*Msp*I (Casals *et al.*, 1990). Three microsatellites, one in intron 8 (IVS8CA) and two in intron 17B (IVS17BTA and IVS17BCA) of the *CFTR* gene, were amplified in a multiplex reaction and were used for indirect analysis of CF. The segregation of alleles for these markers was also used for the confirmation of paternity and for the detection of possible maternal contamination of the fetal DNA sample (Morral and Estivill, 1992). Mutation analysis was performed by polymerase chain reaction (PCR) using specific primers followed by enzymatic digestion when necessary. Depending on the fragment size, polyacrylamide or agarose gel electrophoresis was chosen to visualize them.

In 15 cases, microvillar enzymatic (MVE) analysis (Brock, 1983) was performed by the Biochemistry Service of the Clinic Hospital of Barcelona (Spain) or in the Genetics Unit of the Centro Nationale Transfusione di Sangue of Rome (Italy).

To perform each prenatal diagnosis we followed a flexible scheme, depending on several aspects of the family: (i) the couple's risk of recurrence; (ii) the availability of a previous genetic study; (iii) the informativity for mutations and polymorphisms; (iv) the weeks of pregnancy; and (v) the availability of genetic material from a CF patient in the family.

RESULTS

The distribution of the prenatal diagnosis procedures performed with the 81 samples is shown in Fig. 1. Direct analysis was possible in 36 cases (44·4 per cent); in 24 cases (29·6 per cent) it was necessary to combine mutation analysis with polymorphic markers (in 13 cases with RFLPs and in 11 with microsatellites); in 5 cases (6·1 per cent) mutation analysis and MVE were combined; and in 16 cases (19·8 per cent) only indirect analysis was possible (four using RFLPs, two with microsatellites, four with MVE, two with RFLPs and MVE, and four with MVE and microsatellites).

DNA was successfully obtained from all CVS cases. In one out of 28 AF samples (3·5 per cent) it was not possible to obtain DNA, making it necessary to repeat the amniocentesis procedure. One

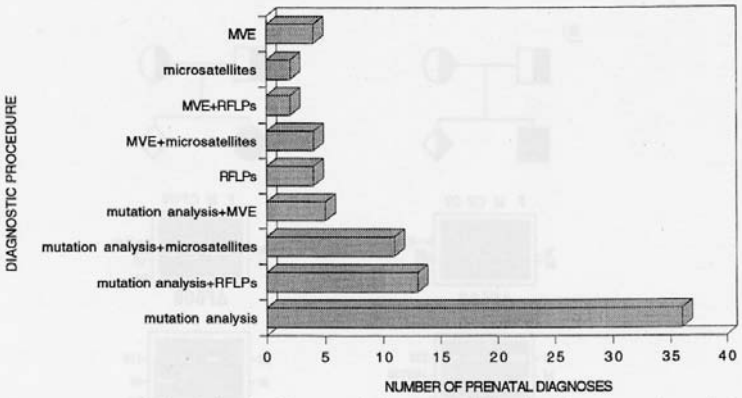

Fig. 1.—Strategy followed in the 81 prenatal diagnoses. MVE=microvillar enzymatic analysis; RFLPs=restriction fragment length polymorphisms

case of spontaneous abortion occurred after chorionic biopsy (1·8 per cent).

The genotype of the CF index cases for which prenatal diagnosis was performed is shown in Table I. Nine different mutations were detected in this series of patients: 621+1G→T, ΔF508, 1609delCA, G542X, G551D, 1949del84, R1162X, W1282X, and N1303K (Morral *et al.*, 1993; Chillón *et al.*, 1994a). The identification of new mutations in the Spanish population allowed us to detect another ten mutations in these families (1811+1·6kbA→G, 711+1G→T, 2869insG, G85E, 2176insC, ΔI507, 3272-26A→G, Q890X, R1066C, and 4005+1G→A). Figure 2 shows two examples of prenatal diagnoses that combine the analysis of two different CF mutations.

Table I—Genotype for 81 cystic fibrosis prenatal diagnoses performed in Spanish families

Genotype	No. of cases
ΔF508/ΔF508	20
ΔF508/G542X	5
ΔF508/G551D	1
ΔF508/N1303K	1
ΔF508/R1162X	1
ΔF508/621+1G->T	1
ΔF508/W1282X	1
ΔF508/1949del84	1
G542X/G542X	1
G542X/N1303K	2
G542X/R1162X	2
ΔF508/unknown	25
G542X/unknown	3
1609delCA/unknown	1
Unknown/unknown	16

These data represent the information available at the time of diagnosis and not the situation at present.

The outcome of all the cases that were diagnosed as being unaffected by CF was a normal child, except in one case. In 1991 we diagnosed a fetus as a CF carrier by the analysis of the ΔF508 mutation (paternal chromosome) and the polymorphic marker J3.11 (maternal chromosome). The child was born with meconium ileum and was diagnosed as affected with CF. Analysis of a blood sample from the child allowed us to prove that there was DNA maternal contamination in the CVS analysed. It should be mentioned that the CVS was sent by courier from another centre. Retrospective information about this CVS indicated that its quality was poor for molecular studies.

Since 1992, we have included the analysis of three *CFTR* microsatellite markers (IVS8CA, IVS17BTA, and IVS17BCA) (Fig. 3) in all prenatal diagnoses. This has allowed us to detect contamination with maternal DNA in three AF samples and in one CVS. In two of these AF samples, the analysis of a new sample allowed genotyping without contamination. In the third AF case, the couple had a low risk (1/240) and the

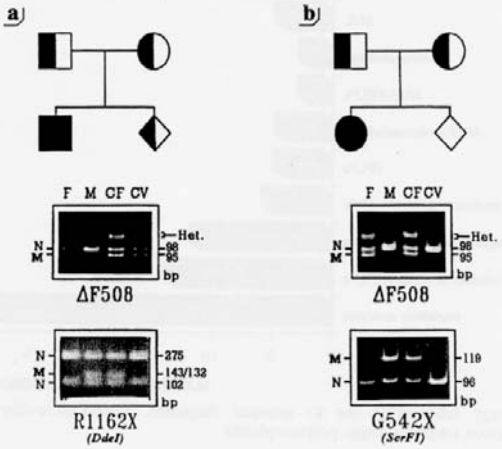

Fig. 2—Direct analysis in two families. (a) Prenatal diagnosis was performed using direct analysis of mutations *Δ*F508 (6 per cent PAGE) and R1162X (2 per cent agarose, previously digested with *Dde*I). The CVS was diagnosed as a carrier. (b) In the second family, prenatal diagnosis was performed analysing mutations *Δ*F508 and G542X (6 per cent PAGE, previously digested with *Scr*FI). The CVS was diagnosed as healthy. (F=father; M=mother; CF=affected child; CV=chorionic villus. Het=heteroduplex bands; N=normal allele; M=mutant allele)

paternal CF allele was not present in the fetus. Finally, in the CVS that showed contamination with maternal DNA, the proportion of contaminating DNA was lower than that corresponding to the inherited allele from the mother (Fig. 4).

The association between haplotypes for microsatellite markers and specific mutations (Morral *et al.*, 1993) allowed us to perform a rapid mutation search in most of the families. Figure 5 shows one family who were at high risk of having a CF child, as they already had a daughter who had died from the disease. Samples for genetic analysis arrived when the mother was 16 weeks' pregnant. The father and daughter were carriers of *Δ*F508, but the mother did not have either *Δ*F508 or G542X (the two most common CF mutations in the population). Microsatellite analysis allowed the identification of haplotype 17-31-13, associated with the maternal CF chromosome. Haplotype 17-31-13 corresponds, among others, to CF mutation R1162X (one of the most common in our population), which was detected in the mother. Thus, we performed a direct prenatal diagnosis on DNA from AF culture by the combined analysis of the two mutations, *Δ*F508 and R1162X.

Of 15 couples with one deceased CF child, we performed direct analysis in ten: *Δ*F508/*Δ*F508 (four cases), *Δ*F508/G542X (three cases), *Δ*F508/R1162X (one case), and G542X/R1162X (two cases).

In 12 prenatal diagnoses (14·8 per cent) the fetuses were predicted to be normal; in 15 other cases (18·5 per cent) the fetuses were diagnosed, by MVE analysis, as 'not affected'; while in 35 cases (43·2 per cent) the molecular diagnosis was of CF carriers and 19 fetuses (23·5 per cent) were diagnosed as affected with CF. These results are in agreement with those expected for an autosomal recessive disease, following the Hardy Weinberg equilibrium.

DISCUSSION

Prenatal diagnosis of CF by direct analysis of the molecular defect of the *CFTR* gene is only

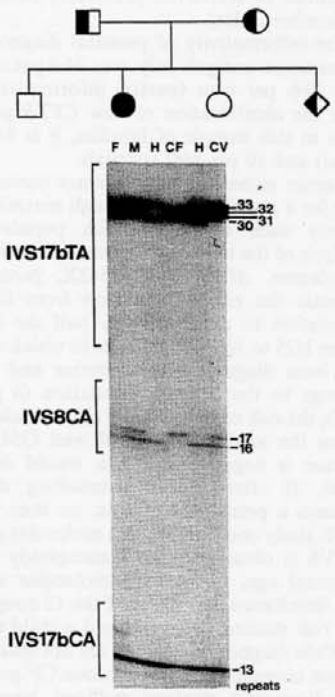

Fig. 3—Multiplex microsatellite analysis of one CF family that was not informative for known mutations in the *CFTR* gene. Informativity was obtained for IVS17BTA and IVS8CA. (F=father; M=mother; H=healthy child; CF=affected child; CV=chorionic villus.) The CVS was diagnosed as a carrier

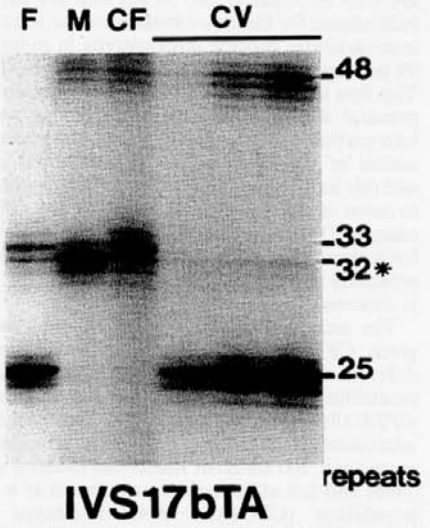

Fig. 4—Microsatellite analysis of IVS17BTA showing contamination of the CVS with maternal tissue. (F=father; M=mother; CF=affected child; CV=chorionic villus.) Different amounts of CV were used (100, 150, and 200 ng of DNA). Low maternal contamination was observed in all of them (allele 32*)

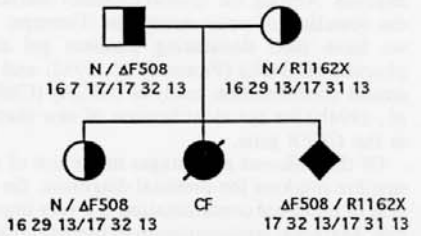

Fig. 5—Pedigree of one CF family with a deceased affected daughter. Results obtained for the ΔF508 mutation and microsatellite analysis (IVS8CA, IVS17BTA, and IVS17BCA) allowed us to identify haplotype 17-31-13 for the maternal CF chromosome and the later identification of mutation R1162X associated with this haplotype. The CVS was diagnosed as affected

possible in those families in which the specific mutations responsible for the disease have been identified. In geographical regions where the frequency of the ΔF508 mutation is about 50 per cent and there is high mutation heterogeneity, direct analysis of the CF defect is only feasible for about two-thirds of cases, after a comprehensive mutation search.

In several cases, the CF family presents during pregnancy and without a previous genetic study. This does not allow rapid screening for all possible mutations, nor the characterization of all exons for unknown *CFTR* mutations in each case. However, informativity for CF in these families should be obtained, both rapidly and with high reliability.

Our current strategy for molecular diagnosis of CF in the heterogeneous Spanish population is based, as a first step, on direct analysis for the two most frequent mutations (ΔF508, 50·6 per cent, and G542X, 8·3 per cent) and indirect analysis using the intragenic markers IVS8CA, IVS17BTA, and IVS17BCA. This combined first analysis allows us to obtain full informativity in all families. Thus, whereas about 30 per cent of couples

are fully informative and 58 per cent are partially informative for these two mutations, the intragenic microsatellites provide informativity in more than 99 per cent of couples (Morral and Estivill, 1992). This first step provides adequate information for a prenatal diagnosis to be performed, as well as information on (i) paternity testing; (ii) contamination of the fetus sample with maternal tissue; and (iii) haplotypes for association with mutations, to assist in the mutation screening of the different samples (Morral *et al.*, 1993). The microsatellite haplotype association with specific mutations is especially useful for couples in which the CF child is deceased.

The second step in the molecular analysis of a given CF family consists of screening for the different mutations associated with the *CFTR* microsatellite haplotypes that segregate with the *CFTR* alleles in each family. Since information on microsatellite haplotypes has been provided for more than 100 different mutations (Morral *et al.*, 1996) and not all mutations are present in a given population (CFGAC, 1994), haplotype data reduce the screening required to a small number of mutations.

The third step consists of a search for unknown mutations in the *CFTR* gene for those cases that have failed in the first and second rounds of analysis. Among the several excellent methods for the detection of point mutations (Grompe, 1993), we have used denaturing gradient gel electrophoresis (DGGE) (Fanen *et al.*, 1992) and single strand conformation analysis (SSCA) (Chillón *et al.*, 1994b) for the identification of new mutations in the *CFTR* gene.

Of the different advantages in the use of microsatellite markers for prenatal diagnosis, the detection of maternal contamination is a very important one. Maternal contamination in prenatal diagnosis has been reported by several authors (Brambati *et al.*, 1992; Rebello *et al.*, 1994). We made an incorrect diagnosis of a CF carrier fetus due to maternal contamination and we observed four further cases of contamination, three in AF samples and one in a CV sample. Thus, even for those cases where the specific mutations that cause the disease have been identified in a given family, we perform a systematic analysis of each family and fetus sample with the *CFTR* microsatellites. In our experience, a tiny amount of maternal material can be present in a few samples, even after cleaning, and the extremely sensitive techniques (radioactive PCR) now in use will reveal the presence of quantities of DNA not previously observed (e.g. by Southern blot).

The informativity of prenatal diagnosis, based on mutation analysis only, was 44·4 per cent (total) and 29·6 per cent (partial informativity). Now, after the identification of new *CFTR* gene mutations in this sample of families, it is 84 per cent (total) and 10 per cent (partial).

Carrier screening for CF is not currently available for a population with a high mutation heterogeneity such as the Spanish population. The analysis of the two most frequent mutations in our population, ΔF508 and G542X, permits us to decrease the risk of a person from the general population to approximately half the initial risk (from 1/25 to 1/60). In couples in which one parent has been diagnosed as a carrier and the other belongs to the general population (*a priori* risk 1/25), the risk of having a CF child would be 1/100. When the analysis of ΔF508 and G542X in the partner is negative, this risk would decrease to 1/240. If after genetic counselling the couple requests a prenatal diagnosis, we then perform a MVE study combined with a molecular analysis. If a CVS is obtained due to aneuploidy risk or to maternal age, we perform molecular analysis of this simultaneously. None of the 12 couples with a low risk studied so far has had a child with CF.

While diagnostic methods are not automatic and robust enough for heterogeneous CF populations, the diagnostic strategy outlined here provides rapid, accurate, and reliable prenatal diagnosis for the majority of couples. This type of approach can also be applied to other genetic disorders where high heterogeneity has been described.

ACKNOWLEDGEMENTS

We thank Dr G. Novelli and Dra E. Casals for MVE analysis, Dr Goossens for DGGE primers, and H. Kruyer for help with the manuscript. This work was supported by grants from the Fondo de Investigaciones Sanitarias de la Seguridad Social (93/0202) and the Institut Català de la Salut.

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Hum Genet (1997) 101 : 365–370

ORIGINAL INVESTIGATION

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High heterogeneity for cystic fibrosis in Spanish families: 75 mutations account for 90% of chromosomes

Received: 3 July 1997 / Accepted: 20 August 1997

Abstract We have analyzed 640 Spanish cystic fibrosis (CF) families for mutations in the *CFTR* gene by direct mutation analysis, microsatellite haplotypes, denaturing gradient gel electrophoresis, single-strand conformation analysis and direct sequencing. Seventy-five mutations account for 90.2% of CF chromosomes. Among these we have detected seven novel *CFTR* mutations, including four missense (G85V, T582R, R851L and F1074L), two nonsense (E692X and Q1281X) and one splice site mutation (711+3A→T). Three variants, two in intronic regions (406-112A/T and 3850-129T/C) and one in the coding region (741C/T) were also identified. Mutations G85V, T582R, R851L, E692X and Q1281X are severe, with lung and pancreatic involvement; 711+3A→T could be responsible for a pancreatic sufficiency/insufficiency variable phenotype; and F1074L was associated with a mild phenotype. These data demonstrate the highest molecular heterogeneity reported so far in CF, indicating that a wide mutation screening is necessary to characterize 90% of the Spanish CF alleles.

Introduction

Mutations in the *CFTR* gene are responsible for cystic fibrosis (CF), a common disorder that affects the digestive, respiratory and reproductive organs (Welsh et al. 1995). More than 600 *CFTR* mutations and a large number of polymorphisms and variants have been reported (http://www.genet.sickkids.on.ca). The incidence of the most frequent mutation, ΔF508 (Kerem et al. 1989), ranges between 30% and 85% of the CF chromosomes in different populations (European Working Group on Cystic Fibrosis 1990) and heterogeneity for other *CFTR* mutations has been observed in several European countries (Claustres et al. 1993; Chillon et al. 1994; Bonizzato et al. 1995; Kanavakis et al. 1995; Hughes et al. 1996b; Estivill et al. 1997). In a previous report (Chillon et al. 1994) we analyzed 486 Spanish CF families and detected 43 mutations that represented 78% of the CF chromosomes. These results indicated the high heterogeneity of this population, but still covered a relatively small proportion of the Spanish CF alleles. In this report we present an update on the spectrum of CF mutations in the Spanish population from a study of 640 CF families. We have identified a total of 75 different mutations representing 90.2% of the CF chromosomes. Among these are seven novel *CFTR* mutations, including four missense (G85V, T582R, R851L and F1074L), two nonsense (E692X and Q1281X) and one splice site mutation (711+3A→T). Three variants, two in intronic regions (406-112A/T and 3850-129T/C) and one in the coding region (741C/T) were also identified.

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Materials and methods

Patients

The patients and their parents were referred to us from several Spanish hospitals. The diagnosis was based on the clinical criteria of CF and at least two positive sweat tests. The clinical information on meconium ileus, hypertrypsinaemia, sweat test values, lung and digestive disease, lung function and clinical radiological scores were obtained for each patient from the clinical centres. Samples were collected from the affected patients and their parents, when available. For the cases in which the patient was deceased, only the carrier parents were studied.

Methods

Genomic DNA samples were isolated from peripheral blood lymphocytes for all members of the 640 CF families using standard methods (Kunkel et al. 1977; Miller et al. 1988). The ΔF508 (Rommens et al. 1990) and G542X (Kerem et al. 1990; Gasparini et al. 1992) mutations were analysed in all patients as they are the most common mutations in the population, 50.6% and 8.0%, re-

Table 1 Seventy-five CFTR mutations identified in 640 Spanish families with cystic fibrosis (CF)

Mutation	Exon/intron	CF alleles	%
ΔF508	E.10	681	53.20
G542X	E.11	108	8.43
N1303K	E.21	34	2.65
1811+1.6kbA→G[a]	I.11	24	1.87
711+1G→T	I.5	22	1.71
R1162X[a]	E.19	21	1.64
R334W[a]	E.7	21	1.64
R1066C	E.17b	14	1.09
1609delCA[a]	E.10	13	1.01
Q890X	E.15	13	1.01
G85E	E.3	12	0.94
712-1G→T[a]	I.5	11	0.86
2789+5G→A	I.14b	11	0.86
ΔI507	E.10	10	0.78
W1282X	E.20	10	0.78
2869insG[a]	E.15	9	0.70
L206W	E.6a	7	0.54
R709X	E.13	7	0.54
621+1G→T	I.4	6	0.47
3272-26A→G	I.17a	6	0.47
R347H	E.7	5	0.39
2183AA→G	E.13	5	0.39
K710X	E.13	5	0.39
2176insC	E.13	5	0.39
3849+10kbC→T	I.19	5	0.39
P205S[a]	E.6a	4	0.31
1078delT	E.7	4	0.31
R553X	E.11	4	0.31
G551D	E.11	4	0.31
1812-1G→A[a]	I.11	4	0.31
CFdel#1[a]	E.4–7/11–18	4	0.31
V232D	E.6a	3	0.23
936delTA[a]	E.6b	3	0.23
1717-8G→A	I.10	3	0.23
1949del84	E.13	3	0.23
W1089X	E.17b	3	0.23
R347P	E.7	3	0.23
del E.3[a]	E.3	2	0.16
R117H	E.4	2	0.16
L558S	E.11	2	0.16
A561E	E.12	2	0.16
2603delT	E.13	2	0.16
Y1092X	E.17b	2	0.16
Q1100P[a]	E.17b	2	0.16
M1101K	E.17b	2	0.16
delE.19[a]	E.19	2	0.16
G1244E	E.20	2	0.16
P5L[a]	E.1	1	0.08
Q30X[a]	E.2	1	0.08
G85V[a]	E.3	1	0.08
E92K[a]	E.4	1	0.08
A120T[a]	E.4	1	0.08
I148T	E.4	1	0.08
711+3A→T[a]	I.5	1	0.08
H199Y	E.6a	1	0.08
875+1G→A	I.6a	1	0.08
1717-1G→A	I.10	1	0.08
L571S	E.12	1	0.08
T582R[a]	E.12	1	0.08
E585X	E.12	1	0.08
1898+3A→G	I.12	1	0.08
G673X	E.13	1	0.08
E692X[a]	E.13	1	0.08
R851X	E.14a	1	0.08
R851L[a]	E.14a	1	0.08
A1006E	E.17a	1	0.08
L1065R[a]	E.17b	1	0.08
F1074L[a]	E.17b	1	0.08
R1158X	E.19	1	0.08
3667del4[a]	E.19	1	0.08
3860ins31[a]	E.20	1	0.08
3905insT	E.20	1	0.08
4005+1G→A	I.20	1	0.08
Q1281X[a]	E.20	1	0.08
Q1313X	E.21	1	0.08
Known mutations (75)		1155	90.23
Unknown mutations		125	9.77

[a] Mutations discovered by the CF group of the Medical and Molecular Genetics Centre – IRO, Barcelona, Spain

spectively (Chillon et al. 1994). To identify other less frequent mutations we took advantage of the haplotypes obtained with three *CFTR* microsatellites (IVS8CA, IVS17bTA and IVS17bCA) analysed by radioactive or fluorescent methods (Morral and Estivill 1992; Shwengel et al. 1994) which provided informativity for each family and facilitated mutation screening (Morral et al. 1996). Routinely, restriction enzyme analysis was used to screen for specific mutations, although in some cases mutagenesis primers which created restriction sites were designed (Haliassos et al. 1989). For totally or partially uncharacterized samples, a wide mutation screening was carried out by multiplex denaturing gradient gel electrophoresis (DGGE) (Costes et al. 1993) for 15 exons and by single-strand conformation analysis (SSCA) (Chillon et al. 1994) for the other 12 exons. For DGGE the DNA fragments were visualised by ethidium bromide staining and for SSCA by silver staining. The abnormal fragments were characterised by sequencing with the DyeDeoxy chain terminator method on an ABI 373A sequencer. Each mutation was analysed in all the available members of the family.

Results

Spectrum of *CFTR* mutations in Spanish CF families

The combined methods in *CFTR* mutations analysis allowed us to characterise 1155 CF chromosomes (90.2%), representing a total of 75 different mutations (Table 1). Only ten mutations have a frequency of 1% and above: ΔF508 (53.2%), G542X (8.4%), N1303K (2.6%), 1811+1.6kbA→G (1.8%), 711+1G→T (1.7%), R1162X and R334W (1.6%), R1066C, 1609delCA and Q890X (1.0%). These ten mutations account for 74.2% of the total CF chromosomes. Eight mutations have frequencies

Table 2 Seven new mutations and three DNA variants in the *CFTR* gene (*IVS* intervening sequence, *DGGE* denaturing gradient gel electrophoresis, *SSCA* single-strand conformation analysis)

Mutations	Exon/ intron	CFTR domain	Haplotype IVS			Detection method
			8CA	17bTA	17bCA	
G85V	E.3	TM1	17	7	17	DGGE
711+3A→T	I.5	–	15	7	17	DGGE
T582R	E.12	NB1	18	37	13	DGGE
E692X	E.13	R	16	46	13	SSCA
R851L	E.14a	–	23	21	19	DGGE
F1074L	E.17b	–	17	31	13	DGGE
Q1281X	E.20	NB2	16	28	13	DGGE
Variants						
406-112A/T	I.3	–		–		SSCA
3850-129T/C	I.19	–		–		DGGE
741C/T	E.6a	–		–		SSCA

that range between 0.5% and 0.9%, representing 6.0% of the CF chromosomes. Twenty-nine CF mutations have frequencies of between 0.1% and 0.4% and cover 7.7% of the CF chromosomes. Finally, 28 mutations were found only once in the population, increasing the proportion of characterised CF alleles to 2.2%.

Segregation studies of the mutations in the CF families allowed us to identify one case of a de novo mutation (L1065R) (Casals et al. 1997). The uniparental inheritance of *CFTR* microsatellite alleles allowed us to detect two large deletions (CFdel#1 and delE.3) (Morral et al. 1993 and unpublished data). In two cases, the direct analysis of mutation R1162X based on the microsatellite haplotype showed a homozygous R1162X pattern in the patient, where only one parent was heterozygous for this mutation and the other was homozygous for the normal allele. This indicates that the patients have a deletion involving exon 19. The new mutations reported here, the method used in their detection and their *CFTR* microsatellite haplotypes are described in Table 2.

New mutations

G85V

An abnormal DGGE pattern in exon 3 due to the nucleotide change G→T at position 386 of *CFTR* determines the missense mutation G85V (glycine to valine). The microsatellite haplotype associated, 17-7-17, is quite frequent in different populations (Russo et al. 1995; Hughes et al. 1996a; Morral et al. 1996). The G85V mutation was identified in a patient who carries the G542X mutation on the maternal CF allele. He was diagnosed when he was 3 years old. At 18 years of age he presents a severe CF phenotype with pancreatic insufficiency (PI) and a FEV1 of 32%, suggesting that G85V is a severe mutation. Another mutation that affects the same codon, mutation G85E, is involved in a milder clinical presentation

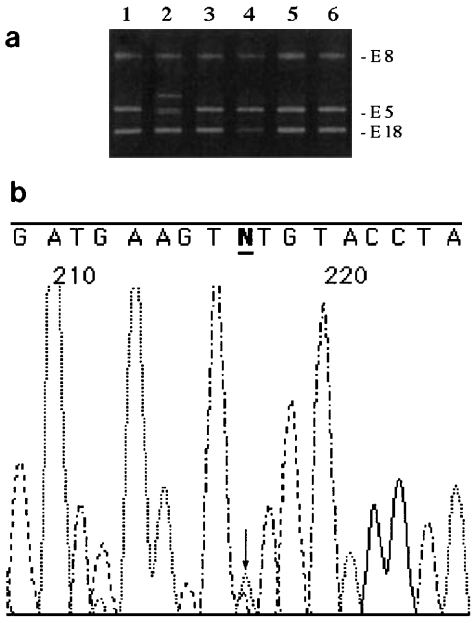

Fig. 1 **a** Multiplex denaturing gradient gel electrophoretic analysis for exons 8, 5 and 18 of the *CFTR* gene. *Lane 2* shows an abnormal pattern for exon 5. **b** Sequencing analysis shows A and T in the third nucleotide of intron 5, corresponding to splice site mutation 711+3A→T

of CF with late-onset pancreatic sufficiency (PS) in 70% of cases (Vazquez et al. 1996). Although a larger number of cases should be analysed, it seems that the glycine change to valine has a more severe effect than that to glutamic acid at this position.

711 + 3A→T

This splice mutation in intron 5 was identified by DGGE analysis (Fig. 1). The CF patient presented the R334W mutation on the paternal chromosome. No other alterations were observed after the complete screening of all the coding and flanking regions of *CFTR*. 711 + 3A → T was associated with the microsatellite haplotype 15-7-17, which was found only once in our CF population. The patient is a 23-year-old woman diagnosed at 19 years due to a recurrent cough and bronchiectasis, mild lung involvement (FEV1 93%), PS and intermittent haemoptysis. The family history showed that the patient had two brothers also diagnosed with CF with PS, who died at 9 and 8 years of age due to respiratory infections.

T582R

An abnormal DGGE pattern in exon 12 was due to a C → G change at position 1877 of *CFTR*, which produced the missense mutation T582R (threonine to arginine). The 18-37-13 *CFTR* microsatellite haplotype associated with T582R is unique in our CF patient population. The patient carries the 1609delCA mutation on the paternal chromo-

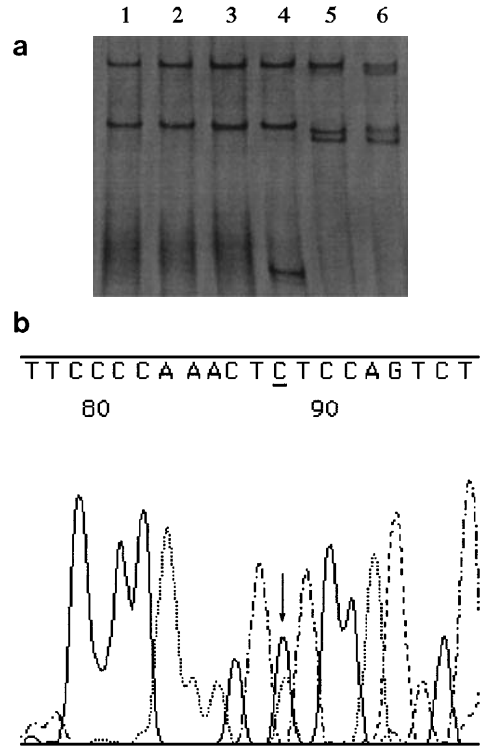

Fig. 2 **a** Single-strand conformation analysis of exon 13a of the *CFTR* gene with three abnormal patterns: *lane 4* (1949del84), *lane 5* (the new mutation E692X) and *lane 6* (K710X). **b** Sequencing analysis of the sample in lane 5 showed the change C → A, which alters the glutamic acid at position 692 to a stop codon (E692X)

some; he was diagnosed with CF with severe lung involvement when he was 37 years old and died 4 years later. The patient was PS. A much better outcome would have been expected if the patient had been diagnosed earlier. Since the patient was diagnosed at a late age, it is likely that mutation T582R causes a milder CF phenotype.

E692X

The SSCA screening for exon 13 detected a G→T nucleotide change at position 2206 of *CFTR*, which gives rise to the nonsense mutation E692X (glutamic acid to the TAG stop codon; Fig. 2). The microsatellite haplotype for this mutation, 16-46-13, is also associated with several other mutations in the CF population (Morral et al. 1996). The patient is 16 years old, diagnosed at 9 years of age, who carries the ΔF508 mutation on the paternal CF allele and presents a severe phenotype (PI, FEV1 77%, colonisation by *Haemophilus influenzae*, nasal polyps and hepatic cirrhosis).

R851L

An abnormal pattern for DGGE of exon 14a detected the missense mutation R851L (arginine to leucine) due to a nucleotide change, G→T, at position 2684 of *CFTR*. R851L was observed in a carrier father whose two sons died due to CF. The consanguinity between the couple suggested that both children were homozygous for this mutation, but this point was not confirmed as the mother was not available for study. The associated microsatellite haplotype 23-21-19 was identified after the study of two children from a second marriage of the father and is present only once in the CF population studied.

F1074L

The F1074L missense mutation (phenylalanine to leucine) due to the nucleotide change T→A at position 3354 in exon 17b of *CFTR* was observed by DGGE analysis. F1074L is associated with microsatellite haplotype 17-31-13 and the mutation was observed in only one family in which the three carrier brothers presented a mild phenotype. The analysis of the IVS8-6(5T) showed this allele *in cis*. Although we have not yet identified the second mutation in this family, we have found F1074L in a congenital bilateral absence of the vas deferens (CBAVD) patient in association with a severe mutation (T. Casals, unpublished data), further confirming the mild effect of F1074L.

Q1281X

The DGGE analysis of exon 20 identified a C→T change at position 3973 of *CFTR*, leading to the nonsense mutation Q1281X (glutamine to the TAG stop codon). The Q1281X mutation is associated with microsatellite haplotype 16-28-13 and has been found in a CF patient with the ΔF508 mutation on the maternal CF allele. The patient was diagnosed at 2 months with pancreatic and lung involvement. At 11 years of age, the patient presents a severe phenotype with bronchiectasis, colonisation by *Pseudomonas aeruginosa* and FEV1 47%.

Two *CFTR* exon deletions

Two samples showed a deletion involving exon 19 and one sample was homozygously deleted for exon 3. These mutations are not completely characterised although they are included in Table 2 as identified CF alleles.

DNA variants

In this study we have identified three DNA variants, 406-112A/T in intron 3 (SSCA), 3850-129T/C in intron 19 by DGGE and 741C/T in the coding region of exon 6a by SSCA, each in a single patient. Other DNA variants and polymorphisms have previously been detected and are reported in previous publications (Chillon et al. 1991, 1992).

Discussion

A geographical distribution analysis of more than 200 CF mutations in several European populations has detected that the Mediterranean region has the highest level of mutation heterogeneity for CF (Estivill et al. 1997). The *CFTR* gene analysis of the Spanish CF population described here confirmed this high heterogeneity, with 75 mutations identified which represent 90.2% of the Spanish CF alleles. Only ten mutations had a frequency of higher than 1%, which account for 74.2% of the CF chromosomes. The level of detection was similar to that obtained for other Mediterranean populations with a relatively low frequency of mutation ΔF508. Claustres et al. (1993) reported 40 mutations accounting for 91% of the CF alleles in the south of France, Bonizzato et al. (1995) reported 22 mutations which account for 90% of chromosomes in the north-east of Italy, and Kanavakis et al. (1995) found 21 mutations for 74.5% of the CF alleles in Greece.

The phenotype-genotype correlation for the new mutations reported in the present work as difficult due to the fact that in several cases only one CF patient for each mutation was observed. However, from the information obtained here, mutations G85V, T582R, E692X and Q1281X can be considered as severe, with lung and pancreatic involvement. For the patient with the 711+3A→T/R334W genotype it is difficult to predict the severity of the 711+3A→T mutation as R334W causes a PS/PI variable phenotype; since two brothers of this patient, also with PS, died at childhood, other genetic factors may explain the clinical variability in this family, as we reported previously (Estivill et al. 1995). The amino acid change in the missense mutation R851L, for which two deceased CF brothers were probably homozygous, should result in a severe mutation. Finally, F1074L was detected in three brothers of the same CF family with a mild phenotype.

We have further confirmed the usefulness of microsatellite haplotypes linked to specific CFTR mutations. While microsatellite haplotypes of mutations G85V and F1074L were also associated with several other mutations, the haplotypes associated with 711+3A→T, T582R and R851L are unique in the CF population studied here, providing a useful tool for mutation analysis (Morral et al. 1996).

The two methods used in this study, SSCA and DGGE, offer a good level of mutation detection, with seven changes detected by DGGE and three by SSCA. The higher level of detection by DGGE is probably due to the higher frequency of the *CFTR* mutations in the exons analysed by this method. In spite of the wide screening performed in the Spanish CF chromosomes, 10% of the CF alleles are still uncharacterised. This is probably due to the efficiency of the methods, the specific regions analysed and the type of mutations. If we regard the 5T allele as a mild mutation, however, we could consider three more chromosomes as characterised (Chillon et al. 1995), leaving 9.5% unidentified.

Our study demonstrates that the Spanish CF population is the most heterogeneous reported so far, indicating that a wide screening is necessary to characterize 90% of the CF alleles. It is important to note that none of the available mutation detection kits are useful in our population as they only detect less than 70% of the CF alleles. The current knowledge of the spectrum of CF mutations in different populations should make it possible to develop specific systems that permit a quick, easy, inexpensive and accurate analysis of 50–100 CF mutations. Otherwise, molecular CF screening programmes with mutation detections of over 95% would hardly be possible in highly heterogeneous CF populations, such as those of most of the Mediterranean countries.

Acknowledgements We thank the ECCACF and M. Goossens for providing primers for multiplex DGGE analysis. This work was supported by grants from the Fondo de Investigaciones Sanitarias de la Seguridad Social (96/2005), the Institut Català de la Salut and the European Union (BMH4-CT96-1364). S.L. is supported from Roche SA and the Associació Catalana de Fibrosi Quística.

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MUTATION IN BRIEF

Paternal Origin of a De Novo Novel *CFTR* Mutation (L1065R) Causing Cystic Fibrosis

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Communicated by Robert Williamson

KEY WORDS: CFTR; paternal origin; cystic fibrosis

INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive disorder with variable clinical expression caused by mutations in the cystic fibrosis transmembrane regulator (*CFTR*) gene (Welsh et al., 1995). In spite of the high number of mutations that have been identified (621 mutations in the CF Genetic Analysis Consortium Newsletter #68; http://www.genet.sickkids.on.ca) in patients with CF and related pathologies (Estivill, 1996), the mutation rate at this locus is estimated to be very low (Morral et al., 1993). Indeed, only one case of de novo mutation in the *CFTR* gene has been reported so far (White et al., 1991).

We have studied 650 CF patients from Spain, where a high heterogeneity for CF mutations has been demonstrated and more than 75 mutations account for 87% of CF alleles (Chillón et al., 1994; T. Casals, unpublished data). In our analysis of a sample of 170 CF chromosomes with unknown mutations, we have identified a new mutation, L1065R, in exon 17b, that was present in the CF patient but not in her father. The patient inherited the ΔF508 mutation on the maternal chromosome. Paternity and fluorescence in situ hybridization (FISH) analyses showed that the CF mutation arose de novo in the paternal allele.

PATIENTS AND METHODS

CF Family (BCN 348)

The patient was a 6-year-old girl diagnosed of CF when she was 1.5 years old. She had lung involvement, pancreatic insufficiency, and high chloride levels (80 mmol/L). Blood samples were obtained on two occasions from the patient, her clinically normal sister, and both parents.

CFTR Gene Analysis

Mutation ΔF508 was analyzed by PCR amplification of exon 10 and the PCR product was visualized after staining with ethidium bromide on 6% polyacrylamide gel electrophoresis (PAGE) (Rommens et al., 1990). Single-strand conformation analysis (SSCA) (exons 1, 2, 4, 6b, 7, 10, 13, 16, 17a, 19, 22, 24) and multiplex denaturing gradient gel electrophoresis (DGGE) (exons 3, 5, 6a, 8, 9, 11, 12, 14a, 14b, 15, 17b, 18, 20, 21, 23) were performed to screen all the coding region of the *CFTR* gene (Costes et al., 1993; Chillon et al., 1994). Three microsatellite markers located within the *CFTR* gene (IVS8CA, IVS17bTA, and IVS17bCA) were analyzed in this family (Morral et al., 1992). Sequencing analysis of an abnormal DGGE pattern detected in exon 17b was carried out (DyeDeoxy™ chain terminator method on an ABI 373A sequencer), using primers 17bi5´ and 17bi3´ (Zielenski et al., 1991). The T→G change at 3326 creates an *Mae*II restriction site, which gives rise to two additional bands of 256 and 208 bp. Restriction analysis of the polymerase chain reaction (PCR) products was used for the study of the different members of family BCN 348 and for a sample of 50 normal chromosomes and 16 CF patients with unknown *CFTR* mutations and the same microsatellite haplotype.

Paternity Study

Seven microsatellite markers located on different human chromosomes (*D6S89*, *ACTBP2*, *SCA1*, *D11S35*, *DRPLA*, *MBP*, *APOC2*) (Genome Data Base) were used to study the paternity in this family.

Received 20 June 1996; accepted 20 June 1996.

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Contract grant sponsor: Fondo de Investigación Sanitaria; Contract grant number: 96/2005; Contract grant sponsor: Institut Català de la Salut.

Cytogenetic and FISH Studies

Peripheral blood cultures were set and harvested after 3 days following standard protocols. G-banding karyotypes of the members of the family showed no cytogenetic abnormalities. Prior to hybridization, DNA from cosmids H-97, containing the sequence pMP6d.9 (*D7S339*), and H-34 (containing the sequence pG2, at the 3′ end within *CFTR*) (Scambler et al., 1987) were labeled with bio-16 dUTP and dig-11 dUTP, respectively, by a standard nick-translation reaction. Hybridization and post-hybridization washes were performed according to previously described protocols (Calonge et al., 1993). Metaphases were studied under an Olympus VANOX fluorescence microscope equipped with the appropriate filter set. Images were analyzed and captured using the Cytovision system (Applied Imaging).

RESULTS AND DISCUSSION

Direct analysis of the ΔF508 mutation showed that the CF patient and her mother were carriers of this amino acid deletion. Microsatellite markers (IVS8CA, IVS17bTA, and IVS17bCA) were studied to obtain informativity about the carrier status of the sister of the CF patient and her parents. Homozygosity was observed in the father's sample for these markers, as well as for several other intragenic and extragenic *CFTR* markers (Morral et al., 1994). Consaguinity between the paternal grandparents was discarded, although they were from neighboring villages.

After the analysis of all the *CFTR* coding and intron/exon flanking regions, only an abnormal pattern in DGGE analysis was detected in exon 17b. Sequencing of exon 17b revealed a T→G change at nucleotide 3326, which predicts a leucine-to-arginine substitution at codon 1065 of *CFTR* (L1065R) (Fig. 1). The abnormal DGGE pattern was found once among 170 CF chromosomes. The T→G change at 3326 creates a *Mae*II restriction site, generating two additional fragments of 256 and 208 bp. The new mutation, L1065R, is associated to haplotype 16–32–13 for microsatellites IVS8CA, IVS17bTA and IVS17bCA (Fig. 2).

In order to follow the segregation of the L1065R mutation in the family members, the four samples were amplified and digested with *Mae*II, but only the sample of the CF patient presented this mutation, suggesting either a sampling error or nonpaternal inheritance of this mutation. New blood samples from the family members were obtained and the study of the L1065R mutation and *CFTR* markers again showed the same results. A study with seven microsatellite markers from different chromosomes confirmed paternity with a probability of higher than 99.9%. Standard cytogenetic analysis showed normal karyotypes in all the members of the family. FISH analysis with cosmids H-97 and H-34 on metaphases of the father showed no evidence of any abnormality in the 7q31 region.

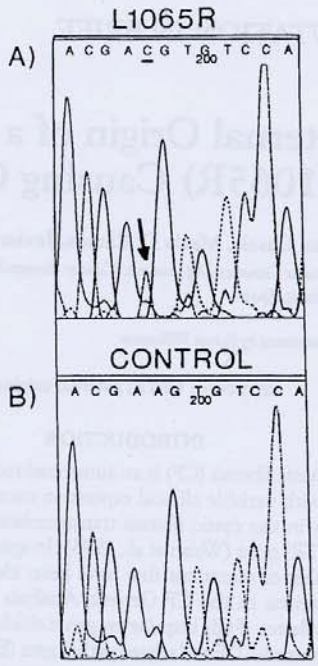

FIGURE 1. **Identification of mutation L1065R in the *CFTR* gene. Sequencing of exon 17b was performed with primer 17bi3′. A:** Partial sequence of exon 17b of the CF patient with mutation L1065R, showing the A→C change on the complementary strand. **B:** Normal control.

The absence of this mutation in the father of the CF patient demonstrates that L1065R has arisen de novo in this family. Although the L1065R mutation was not found in the blood lymphocytes of the father, it could be that his germline presents mosaicism for this mutation, as has been demonstrated in other disorders (Lazaro et al., 1994). However, this analysis was not possible, since the father had been vasectomized several years before, and the possibility of microaspiration of sperm was disregarded. Thus, the state at which this de novo mutation occurred cannot be determined.

While de novo mutations are common in dominantly inherited disorders, such as neurofibromatosis and tuberous sclerosis, among many others, the appearance of new mutations are very uncommon in recessive diseases (Cooper and Krawczak, 1993). Our report is the second case described of a de novo mu-

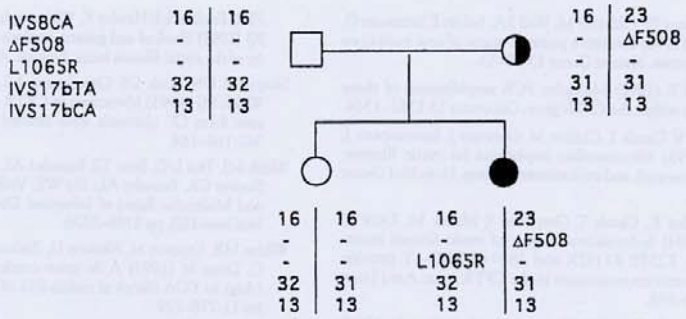

FIGURE 2. Pedigree of the CF family BCN 348 showing the results of microsatellite haplotypes (IVS8CA, IVS17bTA, IVS17bCA) and the two mutations detected (DF508 in exon 10 and L1065R in exon 17b of the *CFTR* gene). –, absence of the mutation.

tation in the *CFTR* gene. The first one was reported by White et al. (1991) and was a new mutation (R851X) arising on the paternal chromosome of a CF patient. Although the only two cases of de novo mutations in the *CFTR* gene described so far have originated on paternal chromosomes, this does not allow us to draw conclusions, but opens the possibility to speculate that there is a higher mutation rate at the *CFTR* gene in paternal gametes. The higher proportion of de novo point mutations in paternal gametes has also been detected in several other genes, such as the fibroblast growth factor receptor-2 (*FGFR2*) gene in Apert syndrome (Moloney et al., 1996). Mutational analysis of individual sperm could confirm this possibility for the *CFTR* gene.

The codon containing mutation L1065R belongs to the second membrane spanning domain (MSD2) of the CFTR protein and is involved in the formation of the chloride channel pore (Sheppard et al., 1993). Thirty-eight mutations have been described in exon 17b of *CFTR* (Mercier et al., 1994; CF Genetic Analysis Consortium http: //www.genet.sickkids.on.ca), encompassing a sequence of 213 nucleotides. Interestingly, nucleotides 3326, 3328, and 3329 each have two different *CFTR* mutations, affecting codons 1065 and 1066. The degree of severity of the amino acid changes affecting these codons varies. Thus, while in some cases the missense mutations are involved in a severe CF phenotype (L1065P, Ghanem et al., 1994; L1065R, this report, and R1066C, Casals et al., unpublished observations), other changes are only associated with male infertility (R1066H, Mercier et al., 1995).

From the genetic counseling point of view, the identification of these de novo mutations in CF families highlights the importance of the segregation studies in identifying the carriers of the mutations in a given family

ACKNOWLEDGMENTS

We thank Dr. M. Goossens for DGGE primers, H. Kruyer for help with the manuscript, and the family reported for their collaboration. This work was supported by grants from the Fondo de Investigación Sanitaria (96/2005) and the Institut Català de la Salut.

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NOTE ADDED IN PROOF

While this article has been in press, two other de novo mutations in the *CFTR* gene have been reported: Hum Genet (1996) 98:119–121 and ECCACF (1996) v.3 n.2.

HUMAN MUTATION 10:387–392 (1997)

RESEARCH ARTICLE

Missense Mutation R1066C in the Second Transmembrane Domain of CFTR Causes a Severe Cystic Fibrosis Phenotype: Study of 19 Heterozygous and 2 Homozygous Patients

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Communicated by Robert Williamson

We report the clinical features of 21 unrelated cystic fibrosis (CF) patients from Portugal and Spain, who carry the mutation R1066C in the *CFTR* gene. The current age of the patients was higher in the R1066C/any mutation group ($P < 0.01$), as compared to the ΔF508/ΔF508 group. Poor values for lung radiological involvement (Chrispin-Norman) and general status (Shwachman-Kulcycki) were observed in the R1066C/any mutation group ($P < 0.005$ and $P < 0.0004$). A slightly, but not significantly worse lung function was found in the R1066C/any mutation group when compared with the ΔF508/ΔF508 patients. No significant differences were detected regarding the age at diagnosis, sweat Cl-values, or percentiles of height and weight between the two groups. Neither were significant differences observed regarding sex, meconium ileus (4.7% vs. 11.1%), dehydration (10.5% vs. 14.7%), or pancreatic insufficiency (PI) (100% vs. 97.8%). The proportion of patients with lung colonization by bacterial pathogens was slightly, but not significantly higher in the R1066C/any mutation group (70.0%), as compared with the ΔF508/ΔF508 group (57.5%). Other clinical complications were significantly more frequent in the R1066C/any mutation patients ($P < 0.02$) than in the ΔF508/ΔF508 group. The two homozygous R1066C/R1066C patients died at the ages of 3 months and 7 years. The data presented in this study clearly demonstrate that the R1066C mutation is responsible for a severe phenotype similar to that observed in homozygous ΔF508 patients. The poor clinical scores and complications of patients with the R1066C mutation are probably related to their slightly longer survival. Hum Mutat 10:387–392, 1997. © 1997 Wiley-Liss, Inc.

KEY WORDS: **cystic fibrosis; R1066C mutation; genotype/phenotype correlation**

INTRODUCTION: R1066C MUTATION STUDY

Cystic fibrosis (CF) is a multisystemic disorder with a wide clinical presentation involving the pulmonary, digestive, and reproductive systems (Welsh et al., 1995). The identification of the CF transmembrane

Received 29 October 1996; accepted 19 January 1997.

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