ORIGINAL INVESTIGATION

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Search for mutations in pancreatic sufficient cystic fibrosis Italian patients: detection of 90% of molecular defects and identification of three novel mutations

Abstract A cohort of 31 cystic fibrosis patients showing pancreatic sufficiency and bearing an unidentified mutation on at least one chromosome was analyzed through denaturing gradient gel electrophoresis of the whole coding region of the cystic fibrosis transmembrane conductance regulator gene, including intron-exon boundaries. Three new and 19 previously described mutations were detected. The combination of these with known mutations detected by other methods, allowed the characterization of mutations on 56/62 (90.3%) chromosomes. Among those identified, 17 can be considered responsible for pancreatic sufficiency, since they were found in patients carrying a severe mutation on the other chromosome. Among these presumed mild mutations, eight were detected more than once, R352Q being the most frequent in this sample (4.83%). Intragenic microsatellite analysis revealed that the six chromosomes still bearing unidentified mutations are associated with five different haplotypes. This may indicate that these chromosomes bear different mutations, rarely occurring among cystic fibrosis patients, further underlying the molecular heterogeneity of the genetic defects present in patients having pancreatic sufficiency.

Introduction

Cystic fibrosis (CF) is the most common severe autosomal recessive disorder among Caucasian populations, where

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the incidence is estimated to be 1 in 2500 live births and carrier frequency 1 in 25 (Boat et al. 1989). The gene responsible for the disease (Kerem et al. 1989; Rommens et al. 1989; Riordan et al. 1989), encodes a 1480-amino acid protein named cystic fibrosis transmembrane conductance regulator (CFTR), which is predicted to function as a cAMP-regulated chloride channel (Anderson et al. 1991). Reduced chloride secretion is at the basis of insufficient hydration of ductural mucus in the airways, reproductive system and pancreas, leading to progressive obstructive damage (The Cystic Fibrosis Genotype-Phenotype Consortium 1993). The clinical expression of the disease is heterogenous but most patients with CF typically present with chronic obstructive lung disease, elevated electrolyte concentration in the sweat and insufficient pancreatic exocrine function (pancreatic insufficiency or PI). Approximately 10–15% of CF patients have pancreatic sufficiency (PS) (Shwachman 1975).

To date, over 470 mutations and sequence alterations have been identified by the CF Genetic Analysis Consortium in regions of the CFTR gene coding for different functional domains of the polypeptide chain. The main mutation causing CF, ΔF508, a 3-bp deletion located in exon 10 in the first nucleotide binding fold (NBF I) (Kerem et al. 1989), has a frequency in patients ranging from 30 to 88% in different populations (European Working Group on CF Genetics 1990).

A correlation between the mutations identified and the clinical symptoms has been observed with pancreas status (Corey et al. 1989; Kerem et al. 1990a), while severity of lung involvement is not so clearly related to the CFTR genotype, suggesting that this phenotype might be modulated by additional genetic or environmental factors (The Cystic Fibrosis Genotype-Phenotype Consortium 1993). Pancreatic sufficient status is genetically determined by mild mutations, mostly being missense, which have been hypothesised to confer a higher residual CFTR activity than the severe ones (Kerem et al. 1989).

Identifying the molecular defects associated with the PS phenotype could be very useful in further clarifying the role of the CFTR protein, and eventually in prognostic

evaluations and establishing care protocols. Furthermore, searching for mutations in the group of patients with PS may reveal the presence of some predominant mutations, which should be preferentially tested.

Data available on the overall CF population, including both PI and PS patients, indicate a high heterogeneity of molecular defects. A few mutations have been so far associated with the PS phenotype (Tsui 1992; Kristidis et al. 1992), but no extensive molecular characterization focused on patients with PS has been reported yet. We have studied a cohort of 31 Italian patients with PS using firstly traditional methods to screen for mutations which predominate in the Italian population [ΔF508, G542X (Kerem et al. 1990b), N1303K (Osborne et al. 1991), 1717-1G→A (Guillermit et al. 1990) and W1282X (Vidaud et al. 1990)], secondly denaturing gradient gel electrophoresis (DGGE) analysis of the entire coding part of the CFTR gene, thirdly testing for the presence of the two mutations [1811+1.2kbA→G (Chillon et al., personal communication to the CF Genetic Analysis Consortium) and 3849+10kbC→T (Highsmith et al. 1994)] located in non-coding portions of the gene, which were not detectable by DGGE, and finally intragenic microsatellites [IVS8/GT (Morral et al. 1991), IVS17b/TA and IVS17b/CA (Zielenski et al. 1991b)] mapping.

Materials and methods

CF patients

DNA specimens were obtained from 31 unrelated Italian nuclear families with a CF child (18 males and 13 females) showing PS. These patients were regularly followed at the Milan CF Center at the Department of Pediatrics, University of Milan. CF diagnosis was based on typical findings of pulmonary CF-related disease (24 cases), on metabolic alkalosis mimicking Bartter's syndrome (3 cases) or on positive neonatal screening (4 cases). Diagnoses were confirmed by a sweat chloride concentration of 60 mmol/l or higher, during Gibson Cooke pilocarpine iontophoresis (Gibson and Cooke 1959), in all but three cases. In the three patients with chloride values in the borderline range (40–60 mmol/l), CF was diagnosed for typical *Pseudomonas aeruginosa* bronchitis and chest radiological features (Stern et al. 1978; Davis et al. 1980). Age at diagnosis varied widely only for patients diagnosed because of pulmonary symptoms (median age 12 years 7 months; range 3 months to 33 years 10 months). Patients' age ranged at sampling from 2.5 to 36.5 years (mean age 17 years 7 months ± 8 years 3 months).

Assessment of clinical features

Pancreatic sufficiency was identified by fecal fat balance (3 days fecal fat collection to calculate the absorption coefficient) (van der Kamer et al. 1949) over 90% in all cases over age of 3 years, by absence of steatorrhea measured with steatocrit (Phuapradit et al. 1981) in the only child under 3 years, and by pancreatic stimulation test (Durie et al. 1984) in three adult patients with sweat chloride in the borderline values.

Pulmonary status was evaluated as follows: (1) chest X-ray scored according to Chrispin and Norman (CN) (1974) (0–38; 0 the best); (2) respiratory function was assessed by the following indexes: forced vital capacity (FVC) and forced expiratory volume in 1 s (FEV_1) (Polgar and Promadhat 1971) expressed as a percentage of predicted values for height and sex; and (3) presence of *P. aeruginosa* chronic lung infection.

Nutritional status was evaluated by the following parameters: (1) weight and height centiles; and (2) Cole's index: percent of ideal body weight predicted for height and sex.

All patients showed a normal growth without pancreatic supplementation.

Mutation detection

Screening for mutations which predominate in our population: ΔF508, G542X, N1303K, 1717-1G→A, W1282X, and of the two intronic mutations 3849+10kbC→T and 1811+1.2kbA→G was carried out as previously described (Ballabio et al. 1990; Friedman et al. 1991; Cremonesi et al. 1991; Vidaud et al. 1990; Highsmith et al. 1994; Chillon et al., personal communication to the CF Genetic Analysis Consortium).

DGGE analysis and direct sequencing

DGGE conditions including GC-clamped primer sequences, gradient denaturant concentration, time and voltage of the electrophoretic separations were as previously described (Fanen et al. 1992; Audrezet et al. 1993) except for those indicated in Table 1. At least one control sample, carrying an already known mutation, was run in parallel with samples under investigation, for each exon being examined. Direct sequencing was performed on asymmetric PCR-amplified templates (Gyllensten and Ehrlich 1988).

Microsatellite analysis

Microsatellite analysis was carried out as indicated elsewhere (Magnani et al. 1994).

Table 1 PCR primers and conditions for denaturing gradient gel electrophoresis analysis of exons 1 and 9 (for exon 9 two different PCR products were analyzed under different conditions in order to detect all possible base changes)

Exon	PCR primers 5´→3´	Annealing temperature (°C)	Denaturing range	Electrophoresis time (h)
1	TAGGTCTTTGGCATTAGGAG (55GC)CCAAACCCAACCCATA CACAC	54	40%–90%	6
9	(35GC)TGAAAATATCTGACAA ACTC CCTTCCAGCACTACAAACTA	45	10%–60%	6
9	(37GC)AACAGGGATTTGGGG AATTA AACTAGAAAAAAAAGAGA	50	10%–60%	6

Results

Screening for predominant mutations

A preliminary screening for mutations being predominant in our population was carried out in our series of patients showing PS, revealing the presence of ΔF508 on 19 (30.6%) chromosomes, 1717-1G→A and G542X on 2 (3.22%). In a previous study the overall frequencies of mutations in the whole sample population referring to our Center had been evaluated on a sample of 1018 CF chromosomes having the following frequencies: ΔF508: 516 (50.7%) chromosomes, G542X: 52 (5.1%), 1717-1G→A: 41 (4.0%), N1303K: 35 (3.4%), W1282X: 14 (1.4%) (our unpublished results).
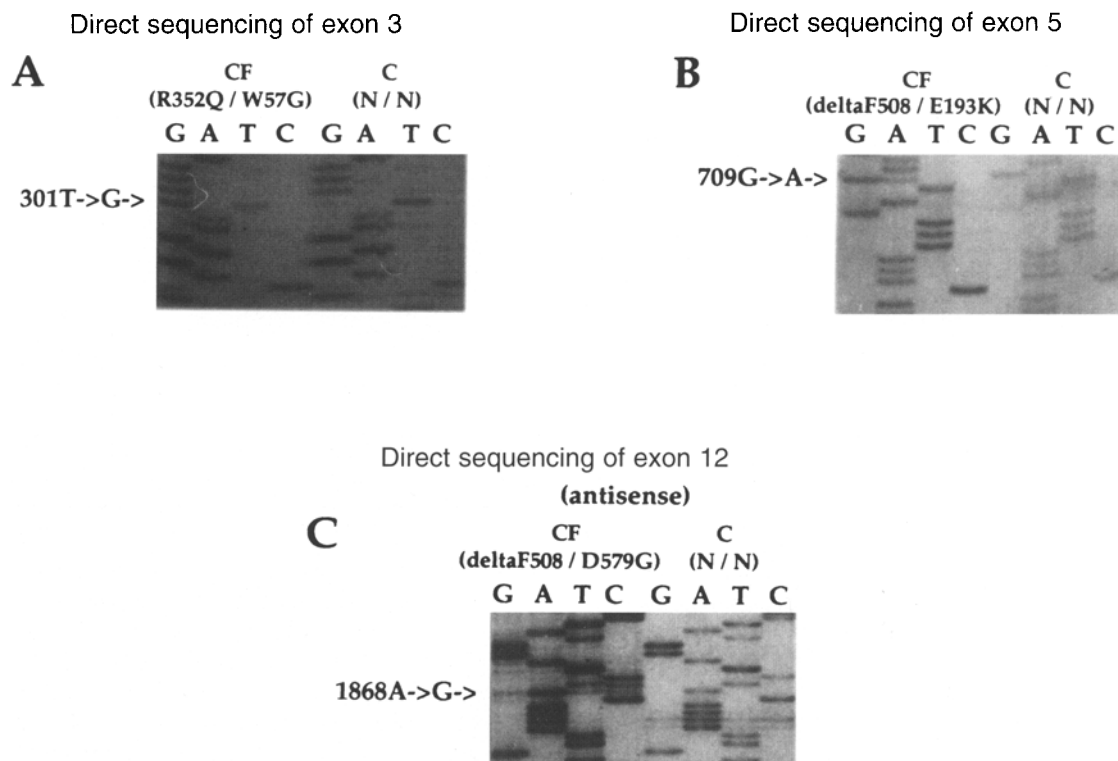
Search for mutations

Direct sequencing of samples displaying altered electrophoretic mobility through DGGE analysis of 27 exons, including intron-exon boundaries of the CFTR gene, allowed the identification of 22 mutations (32 chromosomes). Amongst these, three were previously unreported (W57G, D579G and E193K) (Fig. 1). The remaining 19 included R352Q (Cremonesi et al. 1992) (three chromosomes), G85E (Zielenski et al. 1991a), D1152H (High-

smith et al., personal communication to the CF Genetic Analysis Consortium), R1066H (Ferec et al. 1992), T338I (Saba et al. 1993), 711+5G→A (Gasparini et al., personal communication to the CF Genetic Analysis Consortium), M1V (Cheadle et al. 1993), R334W (Gasparini et al. 1991) (two chromosomes each), 4382delA (Claustres et al. 1993), R1158X (Ronchetto et al. 1992), F1052V (Mercier et al. 1993), G1349D (Beaudet et al. 1991), 1898+3A→G (Cremonesi et al. 1992), S549N (Cutting et al. 1990), 711+3A→G (Petreska et al. 1994), R347P (Dean et al. 1990), 2789+5G→A (Highsmith et al. 1990), R1066C (Fanen et al. 1992) and S1251N (Kälin et al. 1992) (one chromosome each). All mutations altering a restriction site were confirmed by restriction digestion.

The W57G mutation was a T301 to G transversion in exon 3 substituting tryptophan at position 57 with glycine, and was detected in a patient from Northern Italy (Lombardia) bearing the R352Q mutation on the other chromosome. This patient presented with severe respiratory symptoms since childhood, but CF diagnosis was made at the age of 22 years (sweat test 101.74 mmol/l; fat balance 95.45%; severe pulmonary phenotype: FEV$_1$ 17%, FVC 34%, chest X-ray score 33, chronic lung *P. aeruginosa* infection for several years and cor pulmonale). At the age of 27 years, she presented with chronic sinusitis, had been on chronic oxygen supplementation for 2 years and continuous parenteral antibiotic therapy for 8 months. She was on a waiting list for heart-lung transplantation.

The D579G mutation was a A1868G transition in exon 12, substituting aspartic acid 579 with glycine and creating an *Avr*II restriction site. This mutation was found in two patients both carrying the ΔF508 mutation on the other chromosome. The first patient was from Southern

Fig. 1 A–C Direct sequencing of PCR products from three cystic fibrosis patients *(CF)* carrying the W57G **(A)**, E193K **(B)** and D579G **(C)** mutations, in parallel with control samples *(C)* displaying normal sequences *(N/N)*

Direct sequencing of exon 3

A CF (R352Q / W57G)   C (N / N)
G A T C   G A T C

301T->G->

Direct sequencing of exon 5

B CF (deltaF508 / E193K)   C (N / N)
G A T C   G A T C

709G->A->

Direct sequencing of exon 12
(antisense)

C CF (deltaF508 / D579G)   C (N / N)
G A T C   G A T C

1868A->G->

Italy (Puglia), and presented with CF-related symptoms in the first years of life. CF with pancreatic sufficiency was confirmed at 7 years (sweat test 77 mmol/l chloride, fecal fat balance 97.81%). Clinical evaluation at the latest visit (20 years) was as follows: FEV_1 50%, FVC 77%, chest X-ray score 24. Chronic *P. aeruginosa* and *Xanthomonas maltophilia* lung infection occurred since 12 years of age, allergic bronchopulmonary aspergillosis (ABPA), upper right lobe atelectasis and repeated emophtoe since the age of 10 years which needed repeated bronchial artery embolizations. For the second patient carrying the D579G mutation, it was not possible to define the grandparental transmission [grandparents originated from southern (Puglia) and northern Italy (Lombardia-Emilia)]. He presented with pulmonary symptoms at the age of 1 year but, due to previous misdiagnosis (tuberculosis, sarcoidosis and bronchiectasis) and to lack of pancreatic insufficiency, CF was diagnosed at the age of 18 years on the basis of sweat test value (63 mmol/l chloride). PS was confirmed by fecal fat balance (96%). Clinical evaluation at the latest visit (28 years 10 months) was as follows: FEV_1 29%, FVC 52%, chest X-ray score 30. He has had *P. aeruginosa* chronic lung infection since age 18 years 6 months. He is azoospermic.

The third mutation, E193K, was a G709→A transition in exon 5 substituting the glutamic acid 193 with a lysine. It was found in a patient originating from the center of Italy (Abruzzo), and carrying the ΔF508 mutation on the other chromosome, diagnosed at the age of 20 years (sweat test 82 mmol/l). This patient showed PS associated with mild pulmonary disease. Clinical and biochemical assessment at the latest visit (24 years 4 months) were as follows: FEV_1 91%, FVC 99%, CN score 13, fecal fat balance 94%, presence of intermittent *Pseudomonas* infection.

The W57G mutation was not detected on an additional 132 CF and 50 normal chromosomes, D579G on an additional 115 CF and 50 normal chromosomes and E193K on an additional 108 CF and 54 normal chromosomes.

Chromosomes still carrying unidentified molecular defects after DGGE analysis of the whole coding region of the CFTR gene were additionally screened for the two intronic mutations 1811+1.2kbA→G and 3849+10kbC→T, leading to the identification of one (1.6%) chromosome bearing 3849+10kbC→T. In total, mutations were detected on 56/62 (90.3%) chromosomes. Table 2 shows patients' genotypes after the preliminary screening (left side) and at the end of the study (right side).

Microsatellite haplotypes

Five intragenic IVS8/GT, IVS17b/TA and IVS17b/CA microsatellite haplotypes were found in association with the six chromosomes still carrying unidentified mutations: 16-30-13 (two chromosomes), 16-30-14, 16-31-13, 16-28-12 and 16-7-17 (one chromosome each). The two haplotypes 16-30-14 and 16-28-12 were not detected among 220 CF and 220 normal chromosomes (our unpublished

Table 2 Mutations detected in Italian cystic fibrosis (CF) patients having pancreatic sufficiency. (*UN* yet unidentified mutation)

Patient number	Genotype after preliminary screening	Genotype at the end of the analysis
1	UN/UN	M1V/4382delA
2	1717-1G→A/UN	1717-1G→A/R1066H
3	ΔF508/UN	ΔF508/D579G
4	UN/UN	M1V/UN
5	ΔF508/UN	ΔF508/UN
6	UN/UN	T338I/R1158X
7	UN/UN	G85E/711+5G→A
8	UN/UN	D1152H/UN
9	ΔF508/UN	ΔF508/UN
10	ΔF508/UN	ΔF508/3849+10kbC→T
11	UN/UN	711+3A→G/UN
12	ΔF508/UN	ΔF508/F1052V
13	UN/UN	R352Q/W57G
14	UN/UN	1898+3A→G/UN
15	ΔF508/UN	ΔF508/711+5G→A
16	G542X/UN	G542X/D1152H
17	ΔF508/UN	ΔF508/E193K
18	1717-1G→A/UN	1717-1G→A/2789+5A→G
19	ΔF508/UN	ΔF508/G1349D
20	ΔF508/UN	ΔF508/G85E
21	ΔF508/UN	ΔF508/R347P
22	ΔF508/UN	ΔF508/R352Q
23	ΔF508/UN	ΔF508/R352Q
24	ΔF508/UN	ΔF508/S549N
25	G542X/UN	G542X/R1066H
26	ΔF508/UN	ΔF508/T338I
27	ΔF508/UN	ΔF508/R334W
28	ΔF508/UN	ΔF508/R334W
29	ΔF508/UN	ΔF508/S1251N
30	ΔF508/UN	ΔF508/R1066C
31	ΔF508/UN	ΔF508/D579G

results) while the remaining three haplotypes had been found in association with other rare mutations, which were excluded by DGGE analysis in these patients (Table 3).

Discussion

Since the CFTR gene was localized, great efforts have been focused on detecting mutations. To date, patients with PS have not yet been exhaustively analyzed as a distinct group, resulting in scarce information about mutations present in these patients, and only a few mutations have been associated with the PS phenotype. Accordingly, we searched for mutations in a group of patients with PS, originating from different Italian regions, using methods allowing the rapid identification of sequence alterations.

DGGE analysis coupled with direct sequencing in a sample of 31 patients having PS led to the characterization of 22 mutations, including 3 previously unreported. These, in combination with three mutations identified through a preliminary screening for predominant mutations and one intronic mutation, identified molecular de-

Table 3 Microsatellite haplotypes detected in association with yet uncharacterized chromosomes and their distribution among CF and normal chromosomes

Patient number	Microsatellite haplotype			CF chromosomes			Normal chromosomes
	IVS8 GT	IVS17b TA	IVS17b CA	ΔF508	Other mutations	Unknown mutations	
14	16	30	14	0	0	1	0
4	16	31	13	0	2[a]	7	36
11	16	28	12	0	0	1	0
5,8	16	30	13	0	5[b]	11	25
9	16	7	17	0	21[c]	8	33
Total chromosomes analyzed				97	77	46	220

[a] Both chromosomes carry the D1152H mutation
[b] G1349D, R352Q, 1898+3A→G, 4382delA, R334W (one chromosome each)
[c] 1717-1G→A (15 chromosomes), 541delC and 711+5G→A (two chromosomes each), E585X and F1052V (one chromosome each)

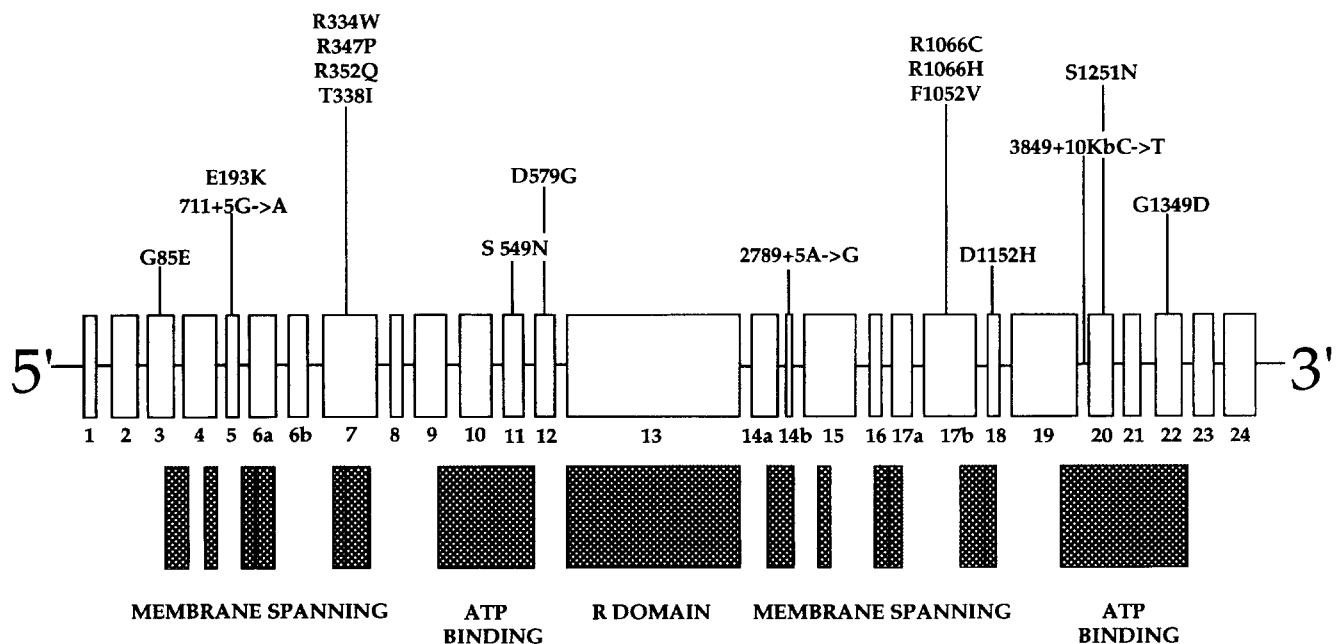fects on a total of 56/62 (90.3%) chromosomes in these patients.

Among the new mutations detected in this study, both D579G and E193K were found in patients compound heterozygous for ΔF508 and presumably cause the mild pancreatic status, being dominant over ΔF508. W57G was found in a patient carrying R352Q on the other chromosome, and we cannot exclude a contribution to the PS phenotype by the W57G mutation. The remaining 19 mutations detected by DGGE in patients with PS had already been described.

DGGE analysis was carried out on the whole coding portion of the CFTR gene. In 7 out of 62 CF chromosomes analyzed at this stage of the study, the molecular

Fig. 2 Localization of the 17 presumed mild mutations, with respect to exons (*blank boxes*, numbered from 1 to 24) and intronic regions. In the lower part, the corresponding putative protein domains are schematically represented (*filled boxes*)

defect was not detected. Search for the only two reported intronic mutations (1811+1.2kbA→G and 3849+10kbC→T) revealed the presence of 3849+10kbC→T, a PS mutation (Augarten et al. 1993), on one chromosome. Only a further analysis at the mRNA level or sequencing of the promoter region could presumably allow the identification of the remaining six still uncharacterized alterations.

In total, among the mutations detected in our PS patients, 17 (D579G, E193K, F1052V, 711+5G→A, G1349D, G85E, R347P, R352Q, S549N, 2789+5A→G, D1152H, R1066H, R334W, T338I, 3849+10kbC→T, S1251N, R1066C) have been detected in compound heterozygosity with a mutation already classified as severe (ΔF508, 1717-1G→A, G542X) and thus can be considered as presumably mild. Of these mutations, seven (G85E, E193K, 711+5G→A, R347P, R334W, R352Q, T338I) are located in the first transmembrane (I TM) domain, five (2789+5A→G, R1066H, F1052V, D1152H, R1066C) in the second transmembrane (II TM) domain, four in the nucleo-

tide binding folds (S549N and D579G in the NBF I, G1349D and S1251N in the NBF II) and one in intron 19 (3849+10kbC→T), further confirming that the milder defects mostly affect the membrane spanning domains with a greater incidence on the I TM (Fig. 2).

The results of this search showed, as expected, a different distribution of classical severe mutations (ΔF508, G542X, 1717-1G→A, N1303K, W1282X) in patients with PS as compared to the overall CF population (37.1% against 67.4%). Moreover, some classical mild mutations, which have been frequently detected in other PS sample populations, are absent (R117H) (Dean et al. 1990) or infrequent (R347P) in our patients. Conversely, other presumably mild mutations such as R352Q (three chromosomes), and G85E, D1152H, 711+5G→A, R1066H, T338I, R334W, D579G (two chromosomes each), are more frequently detected in the PS cohort, accounting in total for 27.4% of chromosomes.

Search for mutations in PS patients should be performed starting with the analysis of exons where most of the mild mutations seem to be located such as exon 7, 5 and 17b in our population, and proceeding with other exons coding for the two TM domains.

Screening for only eight presumed mild mutations (R352Q, R1066H, G85E, D1152H, 711+5G→A, T338I, R334W and D579G) in addition to the predominant four severe mutations (ΔF508, G542X, 1717-1G→A and N1303K), would have allowed the identification of 64.5% of the molecular defects in our patients having PS. This finding is remarkable in view of the lower incidence of severe mutations in patients having PS.

Finally, in 6 out of 62 CF chromosomes analyzed in this study, the molecular defect was not detected. Five different intragenic microsatellite haplotypes were identified in these chromosomes. Among these, two were uniquely detected among CF-PS chromosomes and are not present in 220 CF and 220 normal chromosomes tested. The remaining three, relatively frequent among normal chromosomes, have been found in association with already known mutations, which have been excluded in our sample by DGGE analysis where they were known to be detectable. These findings suggest that mutations on these uncharacterized chromosomes will not be common, further confirming the high heterogeneity of molecular defects in patients with PS.

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