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Complete mutational screening of the *CFTR* gene in 120 patients with pulmonary disease

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Abstract In order to determine the possible role of the cystic fibrosis transmembrane regulator (*CFTR*) gene in pulmonary diseases not due to cystic fibrosis, a complete screening of the *CFTR* gene was performed in 120 Italian patients with disseminated bronchiectasis of unknown cause (DBE), chronic bronchitis (CB), pulmonary emphysema (E), lung cancer (LC), sarcoidosis (S) and other forms of pulmonary disease. The 27 exons of the *CFTR* gene and their intronic flanking regions were analyzed by denaturing gradient gel electrophoresis and automatic sequencing. Mutations were detected in 11/23 DBE (P=0.009), 7/25 E, 5/27 CB, 5/26 LC, 5/8 S (P=0.013), 1/4 tuberculosis, and 1/5 pneumonia patients, and in 5/33 controls. Moreover, the IVS8–5T allele was detected in 6/25 E patients (P=0.038). Four new mutations were identified: D651N, 2377C/T, E826K, and P1072L. These results confirm the involvement of the *CFTR* gene in disseminated bronchiectasis of unknown origin, and suggest a possible role for *CFTR* gene mutations in sarcoidosis, and for the 5T allele in pulmonary emphysema.

Introduction

Cystic fibrosis (CF) is a severe autosomal recessive disorder caused by mutations in the cystic fibrosis transmembrane regulator (*CFTR*) gene. It is characterized by recurrent pulmonary infections, impaired pulmonary function, and disseminated bronchiectasis. The *CFTR* gene may play a role in other isolated pulmonary diseases: disseminated bronchiectasis (Pignatti et al. 1995, 1996; Girodon et al. 1997; Kerem et al. 1997), and allergic bronchopulmonary aspergillosis (Weiner Miller et al. 1996). An increased frequency of *CFTR* gene mutations has also been shown in congenital bilateral absence of the vas deferens (CBAVD) (Chillon et al. 1995; Zielenski et al. 1995), and in other diseases (Estivill 1996). We now present the first thorough analysis of *CFTR* gene mutations performed in a large series of individuals with a variety of pulmonary diseases.

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Materials and methods

Patients

A series of 120 unrelated Italian patients were enrolled in the study: disseminated bronchiectasis of unknown cause (DBE) (23), chronic bronchitis (CB) (27), pulmonary emphysema (E) (25), lung cancer (LC) (26), sarcoidosis (S) (8), bacterial pneumonia (5), lung tuberculosis (TB) (4), and pneumothorax (2). They were admitted or seen in the Institute of Respiratory Diseases of the University of Pavia between 1989 and June 1996, as already described (Pignatti et al. 1995). None of them had clinical or laboratory manifestations of CF or obstructive azoospermia. None had either malabsorption or sinus disease. None had a family history of CF. In addition, 68 healthy blood donors were analyzed.

The subjects participating in this study were divided into four groups: DBE, chronic obstructive pulmonary disease (COPD), unrelated matched control individuals with non-obstructive pulmonary diseases, and normal controls.

The DBE group included 23 patients affected by DBE: 11 males and 12 females. The mean age was 53.7±15.8 years (mean±SD). The diagnosis of DBE was ascertained by high-resolution computer tomography (HRCT) scan appearance or bronchography features. Known and common causes of bronchiectasis, such as primary ciliary dyskinesia, immunodeficiency and α_1-antitrypsin deficiency, were excluded. The pulmonary function tests were performed in 21 out of 23 patients: two of them were unable to undergo the test owing to the severity of their illness and they were assessed only by blood gas analysis. In these patients the mean forced expiratory volume in 1 s (FEV_1) was 58.8±21.3% of the predicted value, whereas the mean forced vital capacity (FVC) was 70.1±22.6% of predicted. The mean age of onset of respiratory symptoms and signs was 22.9±21.2 years. The sweat test was negative for 19 individuals examined; 4 individuals did not undergo the test. Sweat was collected by means of pilocarpine iontophoresis coupled with chemical determination of the sodium concentration. The sodium value (mM) was the medium of at least two deter-

minations. The patients were put on a hyposodic diet 3 days before the test. Familial aggregation of respiratory symptoms was demonstrated in 7/23 patients. One patient was a current smoker, three were former smokers, and the remaining patients had never smoked.

The COPD group included 52 patients, 46 males and 6 females, affected by COPD. The diagnosis was made according to American Thoracic Society (ATS) standards (ATS Statement 1995). In all CB and E patients the sweat test was negative and inherited deficiency of α_1-antitrypsin was excluded. In order better to define the COPD patients, they were further divided into the two major clinical phenotypes included in this group (Snider et al. 1994): CB and E. The CB patients comprised 27 subjects with bronchial mucus hypersecretion and HRCT scan appearance of centrilobular emphysema. Mean age was 63.8±13.1 years. The mean FEV_1 was 60.2±25.2% and the mean FVC was 77.0±17.9% of the predicted value. Ten patients were smokers and 10 ex-smokers; the others had never smoked. Seven patients had familial aggregation with COPD symptoms. Mean age of onset was 50.6±19.7 years. The E patients comprised 25 subjects, with dyspnea and HRCT scan appearance of panlobular emphysema. Mean age was 68.1±10.9 years. The mean FEV_1 was 46.9±26.4% and the mean FVC was 69.5±24.0% of the predicted value. Five patients were smokers and 17 ex-smokers; the others had never smoked. Two patients had familial aggregation with COPD symptoms. Mean age of onset was 56.1±12.6 years.

Also, 45 unrelated matched control individuals with non-obstructive pulmonary disease were enrolled: 32 males, and 13 females. This group included patients with LC (26 subjects), TB (4 subjects), S (8 subjects), bacterial pneumonia (5 subjects) and pneumothorax (2 subjects). These patients were diagnosed, assessed, and treated in the same clinic as the other patients. Mean age was 54.9±17.3 years.

The controls comprised 68 random, unrelated volunteer blood donors with no evidence of pulmonary disease. The mean age was 53.1±18.6 years. Only 33 of the control DNA samples were completely analyzed by denaturing gradient gel electrophoresis (DGGE). While, all of them were analyzed for the IVS8-polyT and the 3849+10 kb C→T mutation.

Mutational analysis

Genomic DNA was extracted from peripheral whole blood samples by standard methods (Sambrook et al. 1989). Polymerase chain reaction (PCR) and DGGE analysis were performed using primers and protocols previously described (Fanen et al. 1992), in multiplex format whenever possible (Costes et al. 1993), on the 27 *CFTR* gene exons and their intronic flanking regions. Mutations detected by DGGE analysis were identified by automatic DNA sequencing. IVS8–5T was analyzed by nested PCR and polyacrylamide gel electrophoresis (Chillon et al. 1995). The 3849+10 kb C→T mutation was detected by restriction enzyme analysis (Highsmith et al. 1994).

Statistical analysis

The frequency of mutations was determined by counts of patients. Differences between proportions were compared by Fisher's exact test, using the EPI Info software (version 5.01). A *P* value of less than 0.05 was considered to indicate statistical significance.

Results

DGGE analysis of the *CFTR* gene was performed in 120 patients with pulmonary disease and in controls. A total of 22 different mutations deemed to be involved in disease predisposition were identified. The mutations were distributed as follows: 7/23 patients with DBE (P=0.046), 2 of whom were compound heterozygotes, 4/27 patients with CB, 1/25 patients with E, 5/8 patients with S (P=0.003), 4/26 patients with LC, and 3/33 controls.

Of these 22 mutations, 14 (R75Q, P111L, R117H, I148T, Y301C, ΔF508, E585X, V754M, L997F, R1066C, M1137V, 3667ins4, D1270N, 4382delA) are listed by the Cystic Fibrosis Genetic Analysis Consortium (CFGAC) as CF mutations (CFGAC website), even if their role in CF disease remains to be proven, as is the case for R75Q, P111L, V754M, L997F, and D1270N. Five mutations (G576A, R668C, R74W, R31C, and I506V) are not thought to be the cause of CF (CFGAC website): three of them (G576A, R668C, and R74W) have been found in CBAVD patients (Anguiano et al. 1992; Chillon et al. 1995; Mercier et al. 1995; Verlingue et al. 1996), R31C was described in a DBE patient (Girodon et al. 1997), and I506V was found in the normal allele in the father of a CF child (Ghanem et al. 1994). Three novel mutations were first identified in this study: D651N, E826K, and P1072L.

All these mutations, except V754M and R31C, affect highly conserved residues among five species investigated: human, bovine, mouse, *Xenopus*, and dogfish (Tucker et al. 1992). The detailed distribution of all the *CFTR* gene mutations detected in the individuals participating in the study is shown in Table 1.

A total of 11/23 (48%) DBE (P=0.009), 5/27 (19%) CB, 7/25 (28%) E, 5/26 (19%) LC, 5/8 (63%) S (P=0.013), 1/4 (25%) TB, and 1/5 (20%) pneumonia patients, and 5/33 (15%) controls, had a *CFTR* gene mutation. Two compound heterozygotes were observed: G576A-R668C/L997F, and ΔF508/L997F. L997F therefore is a recurrent mutation in DBE. L997F was first described in a boy with borderline sweat chloride and features suggestive of cystic fibrosis (CFGAC website).

The IVS8–5T allele was found in 6/23 DBE (P=0.027), 1/27 CB, 6/25 E (P=0.038), 3/26 LC, 1/4 TB, and 1/5 pneumonia patients, and in 5/68 controls. Only one 5T homozygote, a DBE patient, was observed out of a total of 188 subjects examined.

Novel mutations

In this study, three missense mutations in highly conserved residues (Tucker et al. 1992), plus one silent mutation, were discovered. D651N, a G to A transition was detected at nucleotide 2083 in exon 13, which codes for the regulatory domain of *CFTR*. G2083A changes an aspartic acid residue to asparagine: from an acidic to a basic side chain. It was found in a male patient with lung cancer. The mutation destroys a *Taq*I restriction site.

E826K, a G to A substitution, was found at nucleotide 2608 in exon 13. G2608A leads to the change of a glutamic acid to a lysine: from an acidic to a basic side chain. This mutation was found in a female patient with sarcoidosis.

P1072L, a C to T transition was detected at nucleotide 3347 in exon 17b, which encodes part of the second transmembrane domain. C3347T changes a proline to a leucine. It was found in a male patient with chronic bronchitis. The mutation creates an *Alu*I restriction site.

Table 1 The *CFTR* genotypes of pulmonary disease patients and controls. (*CB* chronic bronchitis, *DBE* disseminated bronchiectasis of unknown cause, *E* emphysema, *LC* lung cancer, *S* sarcoidosis, *TB* tuberculosis, *Pnx* pneumothorax)

Clinical status	Total tested	No. of cases	*CFTR* gene mutation[a]	PolyT[b]
DBE	23	1	G576A-R668C/L997F	7/9
		1	ΔF508/L997F	9/9
		1	ΔF508/–	7/9
		1	R1066C/–	5/7
		1	3667ins4/–	5/7
		1	R75Q/–	7/7
		1	M1137V/–	7/7
		1	–/–	5/5
		3	–/–	5/7
		10	–/–	7/7
		2	–/–	7/9
CB	27	1	P111L/–	7/7
		1	R117H/–	7/7
		1	E585X/–	7/7
		1	P1072L/–	7/7
		1	–/–	5/7
		15	–/–	7/7
		6	–/–	7/9
		1	–/–	9/9
E	25	1	R668C/–	7/7
		6	–/–	5/7
		16	–/–	7/7
		6	–/–	7/9
S	8	1	E826K/–	7/7
		1	ΔF508/–	7/9
		1	4382delA/–	7/7
		1	L997F/–	7/9
		1	V754M/–	7/9
		3	–/–	7/7
LC	26	1	I148T/–	5/7
		1	D1270N-R74W	5/7
		1	D651N/–	7/7
		1	Y301C/–	7/7
		1	–/–	5/7
		16	–/–	7/7
		5	–/–	7/9
TB	4	1	–/–	5/7
		1	–/–	7/7
		2	–/–	7/9
Pneumonia	5	4	–/–	7/7
		1	–/–	5/7
Pnx	2	2	–/–	7/7
Controls	68	1	L997F/–	7/9
		1	R31C/–	7/7
		1	I506V/–	5/7
		1	–/–	5/7
		1	–/–	5/9
		23	–/–	7/7
		4	–/–	7/9
		1	–/–	9/9
		2	?	5/7
		23	?	7/7
		10	?	7/9

[a] Minus signs (–) indicate the absence of *CFTR* gene mutations after complete screening by denaturing gradient gel electrophoresis (DGGE); and a question mark (?) denotes that no DGGE analysis was done. The slash (/) indicates compound heterozygosity

[b] The 5T phase relative to the mutations is unknown

2377C/T, a C to T transition, was found in exon 13. It does not change the leucine residue at position 749 (L749L). It does not produce a new splice site (PCGENE software; Staden 1984). It was found in a female patient with emphysema. This last mutation is described here as it is a novel mutation, even if it is not deemed to be involved in the disease.

Polymorphisms

In addition, the following polymorphisms were also found in the patients and controls during the study: 1716G/A (Kerem et al. 1990), in 1 CB, 1 LC, 1 S, 1 PNX patient, and 1 control; 2377C/T (this study) in 1 E patient; 2736A/G (Fanen et al. 1992) in 1 DBE; 3271+18C/T (Romey et al. 1994) in 1 CB; 3690A/G (CFGAC website) in 1 control; 3041–71G/C (CFGAC website) in 2 CB, 1 LC; 4002G/A (Ivaschenko et al. 1993) in 2 CB, 4 LC, and 1 control; 4029A/G (Fanen et al. 1992) in 1 E; 4404C/T (CFGAC website) in 2 CB, 1 E, 1 LC, and 1 pneumonia, and 1 control. Six common polymorphisms were also identified in several patients and controls: 875+40G/T (Fanen et al. 1992), 1540A/G (M470V) (Kerem et al. 1990), 125G/C (5′ untranslated region) (Cutting et al. 1992), TTGA repeat in intron 6a (Gasparini et al. 1991a), 2694T/G (no change at threonine 854) (Zielenski et al. 1991), 4521G/A (no change at glutamine 1463) (Gasparini et al. 1991b).

Discussion

The search for pulmonary disease susceptibility genes is a complex issue, owing to the influence of environmental factors in the pathogenesis of these disorders. Nevertheless, a large number of epidemiologic studies suggest that genetic factors might play a role. Unfortunately, in our study, families in which the disease segregates were not available, therefore only a case-control study was possible.

This study describes for the first time an increase in *CFTR* gene mutations in sarcoidosis and emphysema. It also confirms a significant increase in the frequency of *CFTR* gene mutations in disseminated bronchiectasis patients, in agreement with previous reports from our own group (Pignatti et al. 1995) and from others (Girodon et al. 1997; Kerem et al. 1997), and it indicates the presence of a recurrent mutation in DBE patients.

Only eight sarcoidosis patients were analyzed, therefore the data must be confirmed in a larger sample. This notwithstanding, the five mutations detected in five out of eight sarcoidosis patients are all serious mutations as they are expected to determine changes in the amino acid sequence of the *CFTR* protein. Two deletions (ΔF508 and 4382delA, a frameshift deletion generating a stop codon 15 amino acids downstream) and three missense mutations (V754M, E826K, L997F) were detected. All these mutations affect evolutionarily conserved residues, except V754M, which is, however, thought to be a causative mutation for CF.

In pulmonary emphysema, 24% of the patients carried the 5T mutation, compared with 7% in control individuals. These data indicate that the 5T allele has to be considered as a disease-predisposing mutation in pulmonary emphysema. Also, in DBE of unknown origin, 26% of the patients carried the 5T mutation as already reported (Pignatti et al. 1996). The frequency of the 5T allele in normal individuals in the Italian population was comparable with that reported in other populations (~10%, Kiesewetter et al. 1993; Chillon et al. 1995; Zielenski et al. 1995). The proportion of pulmonary emphysema patients with the 5T allele was lower than that reported for CBAVD (40.2%, Chillon et al. 1995; 51.4%, Zielenski et al. 1995) and for obstructive azoospermia (29.4%, Jarvi et al. 1995). No significant association of *CFTR* gene mutations and 5T was found with the other pulmonary diseases investigated. The fact that the 3849+10 kb C→T mutation is not present in any of the studied subjects is in agreement with our previous observation that the mutation is rare among CF patients in Italy (Bonizzato et al. 1994).

The identification of these *CFTR* gene mutations in the patients indicates the possibility of a follow-up analysis of CF signs and symptoms in compound heterozygotes, in order to detect the possible development of a mild or an atypical form of CF. The presence in these patients of one mutation with a likely role in CF increases their risk of having a child with CF, and it may be considered in genetic counseling. It might be possible to search for common mutations in the partner.

One LC patient had mutations D1270N and R74W, which have been previously described to be syntenic in a CBAVD patient (Mercier et al. 1995). D1270N is now included in the CF mutation list by the CFGAC. Mutation R74W was also syntenic with the 405–46T polymorphism, as previously described (Claustres et al. 1993).

Three missense mutations were detected in 33 controls: L997F, which is present in two DBE and in one sarcoidosis patients; R31C, which was first described in an apparently unaffected 6-year-old child (Ghanem et al. 1994) and next in a DBE patient (Girodon et al. 1997); I506V, which was described in a healthy parent of a CF patient who bore ΔF508 on the other chromosome (Kobayashi et al. 1990).

In the sarcoidosis, emphysema, and disseminated bronchiectasis patients in whom no *CFTR* gene mutations were detected, other genetic and/or environmental factors must be responsible for the disease. Among genetic factors, mutations in other regions of the *CFTR* gene that were not analyzed in this work (e.g. promoter or deep intronic regions), or other genes, should be considered.

These results therefore indicate, at the molecular genetic level, the connection between CF and disseminated bronchiectasis of unknown origin, pulmonary emphysema, and, possibly, pulmonary sarcoidosis. The involvement of the *CFTR* gene in the last disease needs to be confirmed by further studies.

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