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Screening for Non-DeltaF508 Mutations in Five Exons of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Gene in Italy

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Summary

Analysis of exons 10, 11, 14a, 15, and 20 of the cystic fibrosis transmembrane conductance regulator (CFTR) gene by denaturing-gradient-gel electrophoresis (DGGE) allowed the identification of mutations causing cystic fibrosis (CF) in 25 of 109 non-deltaF508 chromosomes, as well as identification of a number of polymorphisms and sequence variations. Direct sequencing of the PCR fragments which showed an altered electrophoretic behavior not attributable to known mutations has led to the characterization of four new mutations, two in exon 11, and one each in exons 15 and 20. Screening for the different mutations thus far identified in our patients by the DGGE analysis and other independent methods should allow detection of about 70% of the molecular defects causing CF in Italy. Mutations located in exons 11 and 20 account for at least 30% of the non-deltaF508 mutations present in Italian CF patients.

Introduction

Following the description of the cystic fibrosis transmembrane conductance regulator (CFTR) gene (Kerem et al. 1989; Riordan et al. 1989; Rommens et al. 1989), reports from different populations have shown that the frequency of the most common mutation (deltaF508), initially reported to be about 70%, varies markedly, ranging from a minimum of 30% observed in Turkey to a maximum of 88% in Denmark (EWGCFG 1990). In Italy, the frequency of deltaF508 evaluated in a sample of 312 unrelated cystic fibrosis (CF) patients equals 53% (329/624) (L. Cremonesi and M. Devoto, unpublished results).

In the meantime, information on the CFTR gene's intron/exon sequences, which is available through the CF Genetic Analysis Consortium, allowed the identification of other mutations, showing that the number of molecular defects causing CF is much higher than that initially predicted on the basis of the haplotype analysis (Kerem et al. 1989). More than 60 mutations and sequence variations have been reported so far to the CF Genetic Analysis Consortium; moreover, although a few of them seem to recur in different populations, the majority are single mutations identified only in individual cases (Cutting et al. 1990*a,* 1990*b;* Dean et al. 1990; Guillermit et al. 1990; Kerem et al. 1990; Kobayashi et al. 1990; Vidaud et al. 1990; White et al. 1990; Schwarz et al., in press).

Different procedures have been proposed which can be used in the search for sequence variations in cloned genes—namely, RNase A analysis of RNA-DNA heteroduplexes (Myers et al. 1985), chemical cleavage of DNA-DNA heteroduplexes (Cotton et al. 1988), single-strand conformation polymorphisms (SSCP) (Orita et al. 1989), and denaturing-gradient-gel electrophoresis (DGGE) coupled with GC-clamps (Myers

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Table 1

Oligonucleotides for GC-clamped DGGE Analysis of Five Exons of CFTR

Exon[a]	PCR Primer (5′→3′)[b]	Size of Amplified Product (bp)
10:		
i5	(GCCCGACCCCCGCGCGTCCGGCGCC)GCTTAGGATGATAATTGGAGGC	432
i3	CATTCACAGTAGCTTACCCA	
11:		
i5	(GTCGCCCCGCTCGGCCCGTCCCCCTGGCGCCCGGCACCCG)CAGATTGAGCATACTAAAAG	271
i3	TAAAGCAATAGAGAAATGTC	
14a:		
i5	(GCCCGCCGGCCCGACCCCCGCGCGTCCGGCGCCCG)GGTGGCATGAAACTGTACTG	286
i3	TGTATACATCCCCAAACTATCT	
15:		
i5	TCAGTAAGTAACTTTGGCTG	390
i3	(GCCCGCCGTCCCGGCCCGACCCCCGCGCGTCCGGCGCCCG)CCTATTGATGGTGGATCAGC	
20:		
i5	(GCCCGCCGGCCCGACCCCCGCGCGTCCGGCGCCCG)TATGTCACAGAAGTGATCCC	302
i3	GACGTACAAGTATCAAATAGCA	

[a] For each exon, the primer at the 5′ end is reported on the first line (i5), and that at the 3′ end is reported on the second line (i3).
[b] GC-clamp sequences are reported in parentheses.

et al. 1987; Sheffield et al. 1989). In particular, the latter approach has been recently proposed for the analysis of mutations of the CFTR gene in a sample of French CF patients (Vidaud et al. 1990). We report here the results of the analysis, based on GC-clamped DGGE and direct sequencing following PCR, that was carried out in a sample of Italian CF patients for the identification of mutations in the CFTR gene. Results of other techniques used to screen for the identification of specific mutations are also reported.

Material and Methods

A total of 109 CF chromosomes carrying uncharacterized mutations were selected for the DGGE screening. All the 109 chromosomes were deltaF508 negative. In addition, a variable number of the same chromosomes had been previously tested, with other methods (ASO and/or digestion with restriction enzymes as described below), for the presence of other known mutations—in particular, 91 for G542X (Kerem et al. 1990), 56 for W1282X (Vidaud et al. 1990), and 96 for R553X (Cutting et al. 1990*b*). For DGGE screening, a first group of 61 chromosomes originated from a corresponding number of compound heterozygous patients who carried an identified mutation (57 deltaF508, two W1282X, one G542X, and one R553X) on one of their two CF chromosomes. An additional subgroup of 48 chromosomes from 24 CF patients who carried unknown mutations on both chromosomes were investigated by using genomic DNA from the patients' parents, following the strategy already described by Vidaud et al. (1990) for both the amplification of individual exons and their analysis through DGGE based on detection of heteroduplexes. The sequences of PCR primers and GC-clamps used for the DGGE analysis are reported in table 1.

After purification through a Centricon-100 ultrafiltration device (Amicon, Danvers, MA), direct sequencing of the amplification products which gave origin to heteroduplexes was carried out by using the Sanger dideoxy-mediated chain termination method (Sanger et al. 1977) with Sequenase version 2.0 (U.S. Biochemicals, Cleveland). Screening for CFTR gene mutations already published or known through the CF Genetic Analysis Consortium was carried out on random samples of CF chromosomes by PCR amplification of specific exons that was followed by digestion with restriction enzymes indicated in table 2 which recognize mutation sites inside the amplification product and, in one case (G542X), by hybridization with the allele-specific oligonucleotides (ASO). Haplotype characterization of CF chromosomes was performed according to a method described elsewhere (Cremonesi et al. 1990).

Table 2

Results of Screening for Known Mutations

Mutation	Exon	Method	No. of Chromosomes With the Mutation[a]	Total No. of Chromosomes Screened[b]	Overall Frequency[c]	Reference
R334W...........	7	*Msp*I	2	198	1.01	X. Estivill, personal communication
R347P............	7	*Hha*I or *Nco*I	4	183	2.19	Dean et al. 1990
G542X...........	11	ASO (DGGE)	15 (4)	176 (18)	9.79	Kerem et al. 1990
S549N............	11	*Dde*I	1	159	.63	Cutting et al. 1990*b*
G551D...........	11	*Hinc*II or *Mbo*I	0	186		Cutting et al. 1990*b*
R553X...........	11	*Hinc*II (DGGE)	5 (1)	186 (13)	3.02	Cutting et al. 1990*b*
1717-1G→A....	11	(DGGE)	(12)	(109)	11.01	Guillermit et al. 1990
S1255X..........	20	*Hin*dIII	0	130		Cutting et al. 1990*a*
W1282X.........	20	*Mnl*I (DGGE)	7 (3)	124 (53)	5.65	Vidaud et al. 1990

[a] Numbers in parentheses are number of mutations found through DGGE.
[b] Only non-deltaF508 chromosomes are indicated. Numbers in parentheses are number of chromosomes analyzed through DGGE and not previously examined for the same mutation.
[c] Calculated by combining the results obtained by DGGE and the additional independent method.

Results

Mutations of the CFTR Gene

The analysis by DGGE of five exons (10, 11, 14a, 15, and 20) of the CFTR gene from 109 CF chromosomes revealed a total of 25 mutant chromosomes corresponding to eight different non-deltaF508 mutations. Of these, four had not been previously identified, while the remaining ones were already known (Cutting et al. 1990*b;* Guillermit et al. 1990; Kerem et al. 1990; Vidaud et al. 1990). Two of the four newly identified mutations (table 3) are located in exon 11, which codes for part of the first nucleotide binding fold (NBF) of the CFTR gene. The first (1784delG), found in two unrelated patients coming from the same region (Lombardia), is a frameshift mutation due to the deletion of a single nucleotide (G) at position 1783 or 1784 of the CFTR gene. As a result, a stop codon is found in position 558 of the corresponding amino acid sequence. This mutation abolishes an *Mnl*I site. The second mutation (Q552X) located in exon 11 is a C-to-T transition at position 1786 which causes the substitution of a glutamine residue in position 552 by a stop codon. This creates a new recognition site for *Hpa*I, starting at nucleotide 1784. The mutation has not been found in an additional sample of 31 non-deltaF508 CF chromosomes which had not been previously analyzed by DGGE. The remaining two previously unreported mutations are located in exons 15 and 20. The first (2909delT) is a frameshift mutation

Table 3

New Mutations Detected by DGGE Analysis and Direct Sequencing

	Haplotype														
Mutation	MetD *Taq*I	MetH *Msp*I	MetH *Taq*I	E6 *Taq*I	W3D14 *Hin*dIII	PT3 *Ban*II	XV2c *Taq*I	CS7 *Hha*I	KM19 *Pst*I	E9 *Msp*I	E4.1 *Msp*I	J3.11 *Msp*I	Mutation on Other Chromosome	Phenotype[a]	Regional Origin
Exon 11:															
1784delG...	1	...	...	...	...	...	2	...	1	1	...	2	R553X	PI	Lombardia
1784delG...	...	...	2	...	...	...	2	...	1	1	...	2	Unknown	PI	Lombardia
Q552X......	...	...	1	1	2	2	1	2	1	2	2	1	Unknown	PI	Venéto
Exon 15:															
2909delT...	1	...	...	...	...	...	1	...	1	1	...	2	Unknown	PI	Lombardia
Exon 20:															
G1244E.....	1	2	2	1	2	2	1	1	1	1	...	1	deltaF508	PI	Sicily

[a] PI = pancreatic insufficiency.

due to the deletion of one of three consecutive T's in positions 2907–2909. As a consequence, a stop codon is encountered in position 941 of the amino acid sequence. The mutation found in exon 20 is a missense mutation due to a G-to-A transition at position 3863 and generates a glycine-to-glutamic-acid substitution in codon 1244 (G1244E). This mutation also destroys a recognition site, starting at nucleotide 3863, for *Mbo*II.

The haplotypes associated with each mutation are reported in table 3. None of these mutations was found in a total of 48 normal chromosomes, including some with the same haplotypes.

The mutations present in the remaining 20 samples which were positive on the DGGE analysis were identified by analogy with the heteroduplexes originated by samples carrying different known mutations and were always confirmed by restriction analysis, ASO hybridization, or direct sequencing. In this way, a total of four G542X, one R553X, and 12 1717–1G→A in exon 11 and of three W1282X in exon 20 could be identified (see table 2).

The results of screening for known mutations on random samples of CF chromosomes by using techniques other than DGGE are reported in table 2. These data together with those obtained through the DGGE screening give an estimate of the frequencies of the non-deltaF508 mutations detected in our population. Since we estimated the frequency of deltaF508 in Italy to be equal to 53%, the overall frequency of the non-deltaF508 mutations found among our patients amounts to 16%.

Polymorphisms of the CFTR Gene

The DGGE analysis allowed identification of two relatively frequent polymorphisms of the CFTR gene. The most frequent one is present in exon 14a (T854T) (Kerem et al. 1990) and can be identified by restriction of the PCR product with *Ava*II. The T-to-G transversion was found in four of 46 deltaF508 chromosomes; in 25 of 102 non-deltaF508 chromosomes; and in six of 48 normal chromosomes. In a group of 11 deltaF508/other heterozygous patients, the nucleotide substitution was present in one of the two chromosomes, but the exact phase could not be established. The association of deltaF508 with both polymorphic alleles is probably due to recombinations between the two sites, located in exon 14a and exon 10 of CFTR.

The second one (M470V) (Kerem et al. 1990) is located in exon 10 and can be detected by restriction analysis of the PCR product by the enzyme *Hph*I. The corresponding A-to-G transition was detected in seven of 113 non-deltaF508 CF chromosomes and in 13 of 48 normal chromosomes but not in 57 CF chromosomes carrying the deltaF508 mutation. In addition, two chromosomes carrying a G-to-A nucleotide substitution in position 1716 (E528E) and one carrying an A-to-G substitution in position 2736 (V868V) were identified.

Discussion

The cloning of the CFTR gene and the subsequent characterization of CF mutations stimulated discussions on the possibility of implementing population-based screening programs for the identification of CF carriers, as recently summarized by Beaudet (1990). The general consensus is that programs should be postponed until knowledge of about 90%–95% of the molecular defects causing CF is achieved and until appropriate pilot studies have demonstrated their effectiveness (Wilfond and Fost 1990). Meanwhile, testing should be offered to relatives of CF patients and to their partners. However, molecular heterogeneity, which is greater than initially predicted, makes it difficult to screen for all the known CF mutations, especially in those countries where the frequency of deltaF508 is relatively low. As an alternative, the preliminary use of a rapid screening method for point-mutation detection might represent a more effective strategy.

In recent years the search for single-base mutations in disease-related genes has taken advantage of the amplification of genomic DNA by PCR coupled with the use of different techniques. The GC-clamped DGGE approach when compared with RNase A and the chemical method, in detecting mutations of exon 9 of the human acid β-glucosidase gene, has been found to be more reliable and effective (Theophilus et al. 1989). The SSCP method has already been used to screen exons 4 and 7 of the CFTR gene, leading to the identification of three new point mutations (Dean et al. 1990). GC-clamped DGGE has been extensively used for the identification of point mutations in human coagulation factor IX (Attree et al. 1989), in factor VIII (Traystman et al. 1990), and, more recently, in the CFTR gene (Vidaud et al. 1990) and, in principle, should be capable of detecting almost all DNA sequence variations (Sheffield et al. 1989). In the present study the strategy based on GC-clamped DGGE applied to the analysis of mutations of five exons of the CFTR gene has led to the detection of eight different

non-deltaF508 mutations (four of which were previously unknown) in 25 mutant chromosomes. The approach we used allowed us to avoid sequencing whenever the PCR product of an exon from a given sample did not show an altered electrophoretic migration on the DGGE analysis. In theory, any known polymorphism or mutation in any exon should be recognizable by the DGGE method alone, thus restricting sequencing only to the analysis of sequence variations previously unknown.

The analysis of only five exons of the CFTR gene does not allow us to exclude that additional sequence alterations located in other exons occur in the same CF chromosomes that carry identified mutations. However, there is little doubt that Q552X, 1784delG, and 2909delT are disease-causing mutations, since all of them cause a premature termination of CFTR synthesis. As for G1244E, it seems that the substitution of a charged amino acid (glutamic acid) for a neutral one (glycine) may be relevant to the protein functioning. In addition, this mutation (located in exon 20, which is part of the second NBF of CFTR) occurs at an amino acid residue which is conserved in 23 of 24 NBFs from different proteins (Riordan et al. 1989), suggesting that it must be important in ATP binding.

The results shown in table 2 indicate that the mutations detected in exons 11 and 20 (which belong, respectively, to the first and second NBF of CFTR) represent about 14% of all the CF mutations in our population. For an individual from our population who does not have a family history of CF, the probability of being a CF carrier would be reduced from 1/25 to 1/77, once the presence of both deltaF508 and these mutations is excluded. The analysis, by GC-clamped DGGE, of those exons in which the highest frequency of mutations is observed might represent an effective method for mutation screening in known-CF-carriers' partners requesting a carrier test. Pilot studies such as the present one, extended to the analysis of mutations in all the exons, are useful in establishing the best strategy to be developed for such testing in different populations.

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