Detection of Five Rare Cystic Fibrosis Mutations Peculiar to Southern Italy: Implications in Screening for the Disease and Phenotype Characterization for Patients with Homozygote Mutations

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Background: The search for the eight most frequent mutations (i.e., ΔF508, G542X, W1282X, N1303K, 1717-1G→A, R553X, 2183AA→G, and I148T) by allele-specific oligonucleotide dot-blot analysis revealed 78% of 396 cystic fibrosis alleles in Southern Italy. The observation of frequent haplotypes on the unidentified cystic fibrosis alleles suggested that a few mutations could account for a large number of unidentified alleles.

Methods: We screened most of the coding sequence of the cystic fibrosis transmembrane regulator gene by denaturing gradient gel electrophoresis to determine the spectrum of these mutations in 68 unrelated cystic fibrosis patients bearing one or both unidentified mutations.

Results: The screening revealed five mutations, R1158X, 711G→T, 4016insT, L1065P, and G1244E, each of which had a frequency of 1.3–1.8% (7% collectively). The 7% increase in the detection rate (85% vs 78%) reduces by >50% the residual risk of being cystic fibrosis carriers for couples who had tested negative by molecular analysis. We therefore designed a second allele-specific oligonucleotide set to analyze the five mutations. Among the patients analyzed, one patient homozygous for the L1065P mutation expressed a mild pulmonary and intestinal form of the disease with pancreatic insufficiency. Two other patients, homozygous for mutations R1158X and 4016insT, both expressed a severe cystic fibrosis phenotype.

Conclusions: Five cystic fibrosis mutations are peculiar to patients from Southern Italy. The method described for their analysis is efficient, inexpensive, and can be semi-automated by use of a robotic workstation. The results obtained in patients from Southern Italy may have an impact on laboratories in other countries, given the large migrations of populations from Southern Italy to other countries in the last two centuries.

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Cystic fibrosis (CF)7 is the most frequent lethal inherited disease among Caucasians, having a prevalence of ~1 in 2500 newborns. Since the cloning of the cystic fibrosis transmembrane regulator (CFTR) gene in 1989 (1–3), >800 mutations have been detected. A few mutations (i.e., ΔF508, N1303K, G542X, and R553X) are frequent worldwide; the other mutations are regional or “private” mutations. Several mutations are peculiar to specific ethnic groups, i.e., W1282X is frequent among Ashkenazi (4),

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7 Nonstandard abbreviations: CF, cystic fibrosis; CFTR, cystic fibrosis conductance regulator; DGGE, denaturing gradient gel electrophoresis; and ASO, allele-specific oligonucleotide.
T338I is typical among Sardinians (5), and 2183AA→G and R1162X are frequent in Northeastern Italy (5).

Consequently, to be able to provide the molecular analysis of CF for diagnostic purposes, laboratories must know the most frequent mutations in an individual’s ethnic group. Mutation mapping is fundamental for “cascade” screenings of CF families (6), because screening programs on general populations are limited by the genetic heterogeneity of the disease.

Similarly, given the migration and genetic mixing that started at the beginning of this century, awareness of CF mutations “peculiar” to each ethnic group is necessary to increase the “detection rate” of CF alleles and to evaluate correctly the residual risk of being a CF carrier after molecular analysis. Finally, analysis of the clinical expression of CF patients homozygous for rare mutations can improve our knowledge of the genotype-phenotype correlation of the disease (7, 8).

In a “pilot” study, we identified a panel of the most frequent mutations in CF patients from Southern Italy (9). Eight mutations led to the identification of ~80% of CF chromosomes. In the same study, we reported that a few CF haplotypes, determined by the study of three polymorphisms, were frequently observed on CF chromosomes carrying an unknown mutation. This observation prompted us to: (a) search for these unidentified mutations by screening most of the coding regions and intronic boundaries of the CFTR gene, using denaturing gradient gel electrophoresis (DGGE); and (b) set up a rapid detection method to analyze the five most frequent mutations identified through the screening.

Patients and Methods

We studied 198 unrelated patients bearing CF, from the Campania (6 × 10^6 inhabitants) and Basilicata (1 × 10^6 inhabitants) regions of Southern Italy at least up to the second generation. These patients represent the whole known CF population of the Basilicata region and 85% of the known CF population of Campania. The diagnosis, based on clinical data, was always confirmed by the sweat analysis of chloride (cutoff, 60 mEq/L). All patients were first analyzed for eight CF mutations, i.e., ΔF508, N1303K, G542X, W1282X, 1717-1G→A, R553X, 2183AA→G, and I148T (9). The 68 patients bearing one or both unknown mutations (88 CF alleles remained uncharacterized) were screened by DGGE for most of the coding regions and intronic boundaries of the CFTR gene (excluding the following exons: 1, 10, 16, and 22). The same patients were also analyzed for four intragenic (i.e., IVS8CA, IVS17bTA, IVS17bCA, and M470V) and two extragenic (i.e., XV2c and KM19) polymorphisms (10–15).

Methods

The eight CF mutations (i.e., ΔF508, N1303K, G542X, W1282X, 1717-1G→A, R553X, 2183AA→G, and I148T) were identified with a semi-automated procedure based on a single multiplex PCR amplification followed by the allele-specific oligonucleotide (ASO) identification we described previously (9). XV2c and KM19 polymorphisms were analyzed by PCR amplification followed by TaqI and PstI digestion, respectively (10, 11). Polymorphisms IVS8CA (12), IVS17bTA, and IVS17bCA (13) were identified by PCR amplification followed by polyacrylamide gel electrophoresis. The DGGE screening of the CFTR exons was performed as described previously (14, 15). The M470V polymorphism was also analyzed by DGGE of exon 10 (14, 15). The CFTR exons displaying a shift in the DGGE pattern were sequenced using the Sanger protocol (16) with an automated procedure in which the four terminator reactions are marked with fluorescent dideoxynucleotides. The fragments were analyzed with the 373A apparatus of Applied Biosystems (Perkin-Elmer).

ASO dot-blot procedure for the analysis of the five “rare” CF mutations

To analyze routinely the five mutations (i.e., L1065P, 711+1G→T, R1158X, 4016insT, and G1244E) we set up a procedure based on a single multiplex PCR amplification followed by ASO dot-blot hybridization. The amplification was performed using the primers shown in Table 1, in a mixture (50 μL) containing the following: 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.5), 2.5 nmol of each of the

Table 1. Primers for a multiplex PCR amplification and internal oligonucleotides for the ASO dot-blot analysis of the five CF mutations newly identified in Southern Italy.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Forward primer (5')</th>
<th>Reverse primer (3')</th>
<th>Wild-type oligo for ASO dot blot*</th>
<th>Mutated oligo for ASO dot blot*</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1065P (exon 17b)</td>
<td>5' TCAAGAAGATGACCACGATG</td>
<td>3' TATACCTATAGAATGCAAGCA</td>
<td>5' ATGGGACACTTCTGTCCT (52 °C)</td>
<td>5' TGGACACCTCCTGTCCT (52 °C)</td>
</tr>
<tr>
<td>4016insT (exon 21)</td>
<td>5' AATGTTCAAGAGAGCTCACA</td>
<td>3' CAAAAGTACCTTGTGCTCCA</td>
<td>5' AGATTTATATTTTTTCCTGGAAC (52 °C)</td>
<td>5' GTATTTATATTTTTTCCTGGAAC (52 °C)</td>
</tr>
<tr>
<td>711+1G→T (intron 5)</td>
<td>5' ATTTCGCTGCTATGCTGCG</td>
<td>3' AACTCCGCGGTCCTCAGGTG</td>
<td>5' TTATGQAAGATGATGTACCAT (52 °C)</td>
<td>5' TTATGQAAGATGATGTACCAT (52 °C)</td>
</tr>
<tr>
<td>R1158X (exon 19)</td>
<td>5' GCCCGAACAATAACCAAGTGA</td>
<td>3' GCTAACAATCCAGCTCCAGG</td>
<td>5' TCCAGATGGGCTATGTCCTGA (52 °C)</td>
<td>5' TCCAGATGGGCTATGTCCTGA (52 °C)</td>
</tr>
<tr>
<td>G1244E (exon 20)</td>
<td>5' GTCAGGGTAAAGGTGTCGA</td>
<td>3' CTTGAGAAAACCTGCACTGGA</td>
<td>5' CCTTGGGAGAAGACTGGA (53 °C)</td>
<td>5' CCTTGGGAGAAGACTGGA (51 °C)</td>
</tr>
</tbody>
</table>

* For each oligonucleotide, the optimal washing temperature of the filter is reported.
four deoxyribonucleoside triphosphates, 20 pmol of each of the two primers, and 1 U of Taq DNA polymerase (Perkin-Elmer Cetus). The PCR conditions were as follows: 10 PCR cycles of 30 s at 94 °C, 30 s from 65 °C to 56 °C, and 30 s at 72 °C; and 25 PCR cycles of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C. After the multiplex PCR amplification, the five mutations were analyzed using ASO dot-blot analysis, using the pair of oligonucleotides shown in Table 1 for each hybridization.

Results

The DGGE screening allowed us to identify 20 different mutations; five of these, i.e., R1158X, G1244E, 4016insT, 711+1G→T, and L1065P, were observed with a frequency >1.0% among 396 CF alleles from Southern Italy.

An example of the multiplex DGGE analysis is shown in Fig. 1. The homozygote (Fig. 1A) and heterozygote (Fig. 1B) patterns of exon 17b are clearly altered; sequence analysis revealed the L1065P mutation. The mutation creates a restriction site for both MnlI and BsiI. The incidence of the L1065P mutation in our series of 396 CF chromosomes was 1.3%. One patient homozygous for L1065P showed a mild pulmonary and gastrointestinal form of CF, with a moderate pancreatic insufficiency and mild liver involvement. The patient (present age, 18 years), born without meconium ileus, was diagnosed at the age of 1 year; the sweat chloride result was 93 mEq/L.

Fig. 2 shows an example of the DNA sequences in a homozygote and a heterozygote patient for the 4016insT mutation (exon 21), compared with the wild-type sequence. In the heterozygote patient, the mutation produced an overlapping of the sequence of the mutated allele with the wild-type allele after nucleotide 4016. The mutation neither creates nor suppresses restriction sites. In the series of 396 CF chromosomes, the 4016insT mutation was observed in seven chromosomes (1.8%), among which was one homozygote patient. The latter, born without meconium ileus, was diagnosed at the age of 2 years; the sweat chloride result was 70 mEq/L. The patient expressed a severe respiratory phenotype with pancreatic insufficiency and cholestasis.

The R1158X mutation was identified through the altered DGGE pattern of exon 19, followed by sequence
600 French CF patients (18). None of them was described during the screening of the cohorts, seem to be peculiar to Southern Italy. In fact, five mutations frequent in Southern Italy. These mutations, although detected sporadically in other population cohorts, seem to be peculiar to Southern Italy. In fact, none of them was described during the screening of the whole coding region of the CFTR gene in a German population (17); only 711+1G→T was reported in <1.0% of 600 French CF patients (18), and 4016insT was reported on a single CF allele in a Welsh subject (19). Mutation R1158X has a frequency of 0.8% in Greece (20); only one allele bearing the mutation has been described in Spain (21), and two alleles have been described in France (22).

Our data confirm some genetic differences in the CF mutations between Southern and Northern Italy (5). Several mutations highly frequent in Northern Italy (R1162X and 711+5G→A) have not been detected in Southern Italy, neither has T338I, which is peculiar to Sardinia (5). On the contrary, none of the five mutations described in the present study has been identified during screening of the whole coding region of the CFTR gene in Northeastern Italy (5, 23). Mutation L1065P has been reported in ~3% of CF chromosomes (24) from Sicily (Southern Italy).

The concordant haplotype obtained for all five mutations among our patients indicates the recent origin of these mutations (25) and excludes a recurrent origin. Different haplotypes have been described only for R1158X, which suggests a recurrent origin (19) or rather, several recombinant events. Mutation R1158X, which has a frequency of 0.8% in the Greek population (18, 20), could have been introduced into Southern Italy by the ancient Greeks who colonized this geographic area. More recently, the mutation could have spread from Southern to Northern Italy and Europe. This hypothesis is reinforced by the analysis of the polymorphism associated to the CFTR gene. We observed the same haplotype in all five alleles bearing the R1158X mutation, i.e., 1, 2, 6, 7, 17 (XV2c, KM19, IVS8CA, IVS17bTA, and IVS17bCA). The chromosome described in Northern Italy has the same haplotype 1,2 for XV2c and KM19 (26). Of the two chromosomes bearing the R1158X mutation described in France, one showed haplotype 2, 2, 16, 7, 17, and the other

| Table 2. Phenotypic features of three CF patients homozygous for rare mutations. |
|---------------------------------|-----------------|-----------------|
| Genotype | 4016insT/4016insT | R1158X/R1158X | L1065P/L1065P |
| Ethnic origin | Southern Italy | Southern Italy | Southern Italy |
| Present age | 23 years | Death at 20 years | 18 years |
| Age at diagnosis | 2 years | 3 months | 1 year |
| Meconium ileus | No | No | No |
| Nasal polyposis | No | No | No |
| Lung involvement | Severe | Very severe | Mild |
| FEV1, % of predicted | 38 | 18 | 62 |
| Liver involvement | Cholestasis | Moderate | Mild |
| Pancreatic insufficiency | Moderate | Moderate | Moderate |
| Sweat chloride | 70 mEq/L | 98 mEq/L | 93 mEq/L |

Discussion
The DGGE screening of the CFTR coding regions revealed five mutations frequent in Southern Italy. These mutations, although detected sporadically in other population cohorts, seem to be peculiar to Southern Italy. In fact, none of them was described during the screening of the whole coding region of the CFTR gene in a German population (17); only 711+1G→T was reported in <1.0% of 600 French CF patients (18), and 4016insT was reported on a single CF allele in a Welsh subject (19). Mutation R1158X has a frequency of 0.8% in Greece (20); only one allele bearing the mutation has been described in Spain (21), and two alleles have been described in France (22).

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| Table 3. Haplotype of CFTR polymorphisms associated with the five mutations listed in Table 1. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| CF mutation | XV2c | KM19 | IVS18CA | IVS17bTA | IVS17bCA | M470V |
| R1158X | 1 | 2 | 16 | 7 | 17 | M |
| L1065P | 1 | 1 | 16 | 30 | 13 | V |
| 711+1G→T | 1 | 1 | 16 | 25 | 13 | V |
| 4016insT | 2 | 1 | 16 | 30 | 13 | V |
| G1244EG→T | 1 | 1 | 16 | 34 | 13 | V |
showed haplotype 2, 2, 16, 45, 13, suggesting the recurrent origin of the mutation or a recombinant event (18, 22). The only difference between the first French patient and the subjects in our study is the XV2c dimorphic locus, i.e., the one most distant from the CFTR gene. A recombinant event between the XV2c locus and the CFTR gene is not inconceivable. The mutation detected in the second French patient could derive from a second, more recent, recombinant event within the CFTR gene, between exons 8 and 19.

The mutation detection rate of 85% of CF alleles with the analysis of only 13 CF mutations is surprising considering the genetic heterogeneity of the population of Southern Italy (5). In Spain (21), >40 CF mutations identify ~78% of CF alleles, whereas in France, 47 different mutations identify 86% of CF chromosomes (18). The presence of several mutations peculiar to Southern Italy could depend on the high rate of consanguinity among CF carriers from our regions, as is suggested by the high incidence of CF patients homozygous for rare CF mutations.

The possibility of identifying 85% of CF chromosomes through a rapid molecular analysis allows us to estimate the residual risk of being carrier or of having a CF-affected child in couples for whom molecular analysis for CF was negative (see Table 4). In fact, starting from an a priori risk of being a CF carrier of 1:25 (4%), the negative molecular analysis for CF mutations reduces the risk of being a carrier to 1:112.5 (0.88%) if the panel of mutations has a detection rate of 78% (4% of an a priori risk × 22% risk of carrying an unidentified mutation). The risk becomes 1:167 (0.60%) if the panel of mutations has a detection rate of 85% (4% of an a priori risk × 15% of risk of carrying an unidentified mutation). Consequently, the risk of having a CF child for couples for whom molecular analysis for the CF mutation was negative (see Table 4) is 1:50 625 if the detection rate of the mutations analyzed is 78% (1/112.5 × 1/112.5 × 1/4) and becomes 1:111 556 (1/167 × 1/167 × 1/4) at a detection rate of 85%. Similarly, if one member of the couple is a CF carrier and the other is negative by molecular analysis, the risk of having a child affected by CF is 1:450 (1/112.5 × 1/4) if the test has a detection rate of 78% and 1:668 (1/167 × 1/4) if the detection rate is 85%. Consequently, on the basis of these findings, cascade screening can be planned in the families of CF patients (6). The ASO dot-blot procedure described is very easy and efficient because it is based on a single multiplex amplification, which can be semi-automated with the use of a robotic workstation (9), allowing the analysis of large series of DNA samples. Using this procedure to analyze eight CF mutations, we have made >1000 molecular CF diagnoses of homozygote or heterozygote CF subjects over the last 4 years. Similarly, the ASO analysis of the five new mutations gave unequivocal results in all CF patients that had been characterized by DGGE and sequenced previously and in all 100 control alleles. Furthermore, we now routinely screen CF patients for the 13 mutations and have identified several other alleles (data not shown) bearing the five new mutations in both CF patients and subjects affected by congenital bilateral agenesis of the vasa deferentia.

This study confirms that polymorphism analysis is a valid procedure with which to assess the genetic heterogeneity of a population with respect to a specific gene, and to evaluate the recurrent origin of a mutation (22, 23, 27). Similarly, we confirm the very high potential of DGGE as a tool for screening large genes for unknown mutations (14, 15).

The phenotype analysis in the three homozygous patients confirmed, in this case, the phenotype predicted by the molecular analysis. The patient homozygous for R1158X (a nonsense mutation) and the patient homozygous for 4016insT (a frameshift mutation) showed very severe expression of CF (because the synthesis of the wild-type protein was suppressed) compared with the patient homozygous for L1065P, a missense mutation associated with the synthesis of a protein with a single amino acid substitution.

In conclusion, the five so-called rare CF mutations described here seem to be peculiar to Southern Italy. Their analysis, added to the analysis of the eight most frequent

**Table 4. Risk prediction for having CF-affected children for a couple after molecular analysis of CF mutations.**

<table>
<thead>
<tr>
<th>Number of known mutations screened</th>
<th>One mutation is found</th>
<th>No mutations are found</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>1:450 0.22%</td>
<td>1:50 625 0.0020%</td>
</tr>
<tr>
<td>13</td>
<td>1:668 0.15%</td>
<td>1:111 556 0.0009%</td>
</tr>
</tbody>
</table>

Fig. 3. ASO dot-blot analysis of five CF mutations. Mutations were hybridized with the wild-type oligonucleotide (left) and with the mutated oligonucleotide (right). The numbers 1 to 4 refer to single individuals for each of the tested mutations. For R1158X (A) and L1065P (B), 1 and 2 are heterozygotes for the mutation, 3 is a homozygote for the mutation, and 4 is a healthy control. For 4016insT (C), 1 and 2 are healthy controls, 3 is a homozygote for the mutation, and 4 is a heterozygote for the mutation. For 711+1G→T (D) and G124E (E), 1 and 2 are healthy controls, and 3 and 4 are heterozygotes for the mutation.
CF mutations (9), allows us to detect 85% of CF alleles from these regions and to calculate a very low residual risk of having a child affected by CF for couples for whom molecular analysis of the 13 mutations was negative. The use of an additional multiplex PCR associated with a semi-automated ASO dot-blot analysis represents a step forward in the strategy to eradicate the disease from the regions studied. The identification of several rare homozygotic mutations adds useful information to the genotype-phenotype correlation for this disease. Lastly, the results obtained in CF patients from Southern Italy may have an impact on laboratories in other countries, given the large migrations of populations from Southern Italy to other regions of Italy (i.e., Northern Italy) or to countries (i.e., Switzerland, Germany, and North and South America) in the last two centuries.

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References