Detection of mutations in Cuban cystic fibrosis patients

RESEARCH

✍ Teresa Collazo, Yaixa Piloto, Yulia Clark, Ana María Bofill, Manuel Gómez, Yadira Hernández

National Center of Medical Genetics, ISCM-Havana, Cuba
Ave. 30 Esq. 146 No. 3102, Playa 16, Havana, Cuba
E-mail: tcollazo@infomed.sld.cu

ABSTRACT

Cystic Fibrosis (CF) is the most common autosomal recessive disease in Caucasian populations, with an incidence of 1 out of 5000 newborns in Cuba. Although cystic fibrosis transmembrane conductance regulator (*cftr*) gene was cloned and the mutation of this gene responsible for most CF cases, F508del was already identified by 1998, more than 1400 additional *cftr* mutations have been described. The mutations for the *cftr* gene among the Cuban CF patient population are highly heterogeneous. Therefore, we have used two screening techniques, denaturing gradient gel electrophoresis (DGGE) and single strand conformation polymorphism (SSCP) in order to detect and identify *cftr* sequence changes in this population. In the present study, 9 different mutations were detected in our patients, together with 4 previously described nucleotide sequence polymorphisms.

Keywords: Cystic Fibrosis, Single-Strand Conformation Polymorphism, Denaturing Gradient Gel Electrophoresis

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RESUMEN

Detección de mutaciones en pacientes cubanos con fibrosis quística. La fibrosis quística es la enfermedad autosómica recesiva más frecuente en poblaciones caucásicas. En Cuba, 1 de cada 5000 recién nacidos están afectados. El gen regulador de la conductancia transmembranal de la fibrosis quística (*ctfr*) fue clonado en 1989 e identificada la mutación principal F508del. Desde entonces más de 1400 mutaciones diferentes en el gen *cftr* han sido descritas. La población cubana es muy heterogénea para las mutaciones del gen *cftr*. En el presente estudio se emplearon dos técnicas de tamizaje: electroforesis en gel de gradiente desnaturalizante (DGGE) y polimorfismo conformacional de simple cadena (SSCP) para la detección de cambios en la secuencia del gen *cftr*. Se detectaron 9 mutaciones diferentes en los pacientes estudiados y 4 cambios polimórficos en la secuencia nucleotídica, previamente descritos.

Palabras clave: fibrosis quística, polimorfismo conformacional de simple cadena, electroforesis en gel de gradiente desnaturalizante

*I*ntroduction

Cystic fibrosis (CF) is the most common autosomal recessive disease in Caucasian populations [1]. In Cuba, it reaches an incidence of approximately 1 out of 5000 newborns [2], and therefore it is regarded as a public health problem.

This disorder is caused by the presence of mutations in the cystic fibrosis transmembrane conductance regulator gene (*cftr*) [3]. Although more than 1400 *cftr* mutations leading to CF have been described, a single mutation, F508del, accounts for 67% of the CF alleles worldwide, followed by the remaining alleles at markedly lower frequencies [4]. The number of mutations-excluding F508del-reaching frequencies from 0.1 to 3% is not larger than 20, and most of the other alleles constitute rare variants which have been reported only for one or two families [5].

Variability in the number and incidence of *cftr* mutations according to the ethnicity and geographical location of every population is evident. While it is possible to identify more than 97% of the CF alleles in genetically homogeneous populations (such as those of Ashkenazi Jews [6] and Hutterites [7]) by the analysis of a small number of mutations, screening studies using more than 40 CF mutations in much larger and heterogeneous populations such as those of Spain [8] or Italy [9] have reached an allele coverage of only 74 to 78%.

The Cuban population, similarly to those of southern Europe, is highly heterogeneous in regard to *cftr* mutations. A study screening for F508del and 5 of the most common remaining mutations only identified 55.5% of mutant alleles in Cuban patients [10]. Although the information provided by this study confirmed the diagnosis of many of these patients and allowed the identification of carriers and afflicted individuals among their relatives, direct molecular analysis has not provided useful information yet for family planning advice in a significant proportion of the cases.

A number of techniques have been developed for the detection of small nucleotide changes, such as single-base substitutions, deletions, or insertions. One of the early applications of two of the most used methodologies for this purpose, denaturing gradient gel electrophoresis (DGGE)[11] and single-stranded conformational polymorphism (SSCP) heteroduplex analysis [12] was precisely the extensive characterization of the *cftr* gene [13, 14].

The objective of this work was, therefore, to standardize and employ the techniques of DGGE and SSCP for a better characterization of unidentified *cftr* mutations in Cuban patients, mapping the mutations to specific exons and identifying them in order to improve the allele coverage of the molecular diagnosis of CF in our country.

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✍ Author of correspondence

Materials and methods

Twenty-three cases were randomly selected from the Cuban National Cystic Fibrosis Registry, 12 from the western and 11 from the eastern region of the country. These cases corresponded to patients with a clinical diagnosis of CF where it had not been possible to identify the genetic mutation responsible for the disorder. Additionally, 110 patients were used for the analysis of exon 10.

DNA samples were obtained by salting-out [15] from 10 mL of peripheral blood containing EDTA (56 mg/mL) as anticoagulant.

SSCP technique was used for the analysis of exons 6a, 7, 10, 13a, 17a, 2, 4, 6b, 19, 1, 16, 22, 24, 13b and 10 for the CFTR gene, using the oligonucleotide primers reported by Liechti-Gallati *et al*. [16]. Amplification reactions contained 100 ng of DNA, 15 pmol/mL of each primer, 1 mM dNTPs, 1.5 mM $MgCl_2$, and 1 U of Taq DNA polymerase in a final volume of 25 µL, and used a cycling profile consisting of an initial denaturation step of 3 min at 94 °C followed by 35 cycles of 20 s at 94°C, 30 s at 55°C and 30 s at 74°C, followed in turn by a final extension step of 5 min at 74°C. At the end of the amplification, 1 ?L of the reaction was mixed with 3 iL of H2O and 3.5 mL of a bromophenol blue solution, and 10 mM sodium hydroxide; this mixture was denatured for 1 h 30 min at 98°C, quickly placed on ice to slow down renaturation, and loaded into a pre-made acrylamide gel, electrophoresing the samples at 600 V for a time of 80 to 120 min (depending on the specific exon under analysis). Each electrophoretic run included a positive control consisting of a sample with an already identified mutation in the specific exon under study. DNA was visualized by silver staining.

The remaining exons were studied by DGGE, performing multiplexing in all cases and, as before, including a positive control with a known mutation mapped to the specific exon under analysis (Table 1). The oligonucleotide primers used in this case were those of Fanen *et al.* [17]. The DNA was visualized by ethidium bromide staining and fluorescence under UV light.

Results and discussion

After the analysis of all exons by SSCP and DGGE, in every case where a variation was detected during electrophoresis, it was furtherly examined in order to identify the underlying mutation or polymorphism.

A total of 106 variations were detected in exon 10 of *cftr* gene, of which 25 corresponded to mutation F508del (7 in homozygosis and 18 in heterozygosis), 2 to patients carrying the I507del mutation, and 3 to anomalous electrophoretic profiles representing two unidentified mutations or polymorphisms. In the latter case, it was necessary to sequence such samples in order to identify specific mutations (Figures 1 and 2).

Other polymorphisms identified in exon 10 are 1540A/G, 1525-61 G-A and 1576 G/A, already described during the study of Spanish populations among others [18]. Polymorphism 1540A/G was found in 56 of the 220 analyzed chromosomes (25%) and was homozygotic for 4 of those patients; the second allele in the heterozygotic samples was F508del in 8 cases. Polymorphism 1525-61 G-A was detected in 16 chromosomes (7.3%) and polymorphism 1576 G/A was detected in only 2 chromosomes (0.9%) (Figure 3).

The profile of one of the electrophoretic variants analyzed for exon 4 (Figure 4) did not coincide with

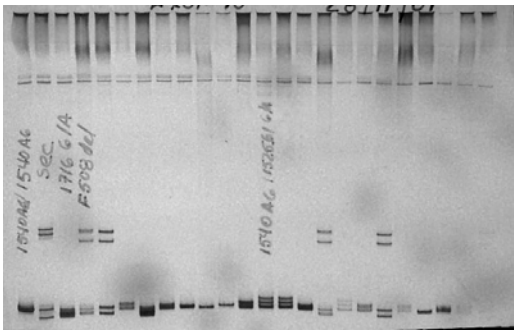

Figure 1. Results of the analysis by SSCP of exon 10, using 12% acrylamide gel electrophoresis. From right to left: Lane 1, patient homozygotic for polymorphism 1540AG; lane 2, anomalous pattern to be sequenced for identification; lane 3, polymorphism 1716A/G; lane 4 corresponds to the heterozygotic positive control containing mutation F508del. Polymorphisms 1540A/G and 1525-61 can also be seen in lanes 13, 14 and 17; lane 16 contains an F508del heterozygote, and lanes 5 and 19 represent unidentified anomalous patterns. The remaining lanes contain samples without mutations in exon 10 from CFTR.

Table 1: Representation of multiplexes A, B, C, D and exon 21 by DGGE

Multiplex	Exons	PCR Conditions	Electrophoresis conditions
Multiplex A	11, 14b, 17b	94 °C-5 min, 94 °C-, 1 min, 50 °C-1 min, 72 °C-2 min, 72 °C-, 7 min (40 cycles)	Gradient: 10-60% Time: 150 min Voltage: 180V
Multiplex B	14a, 15, 20	94 °C-5min, 94 °C-, 1 min, 55 °C-1 min, 72 °C-2 min, 72 °C-7 min (40 cycles)	Gradient: 10-60% Time: 270 min Voltage: 180V
Multiplex C	3, 12, 23	94 °C-5 min, 94 °C-, 1 min, 55 °C-1 min, 72 °C-2 min, 72 °C-7 min (40 cycles)	Gradient: 10-60% Time: 150 min Voltage: 180V
Exon 21	21	94 °C-5 min, 94 °C-20 s, 58 °C-30 s, 72 °C-30 s, 72 °C-7min (35 cycles)	Gradient: 10-60% Time: 270 min Voltage: 180V
Multiplex E	5, 8, 18	94 °C-5 min, 94 °C-, 1 min, 50 °C-1 min, 72 °C-2 min, 72 °C-7 min (40 cycles)	Gradient: 10-60% Time: 150 min Voltage: 180V

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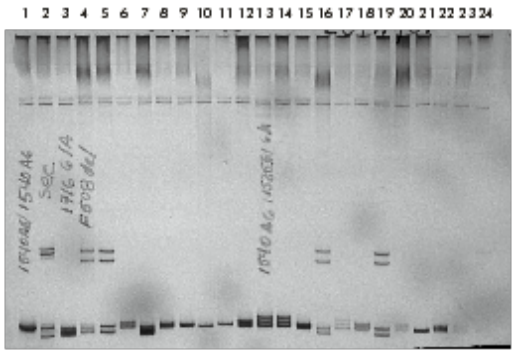

Figure 2. Results of the analysis by SSCP of exon 10, using 12% acrylamide gel electrophoresis. Lane 11 corresponds to the I507del positive control. Lane 7 (from right to left) represents an unidentified pattern; lane 15 presents a pattern matching that of the I507del mutation, and lane 17 contains the pattern corresponding to mutation F508del.

the control used for these experiments. DNA sequencing of this sample revealed the presence of mutation Y109C, which is frequent in Switzerland but was not found in a study of the Spanish population [9]. The patient carrying Y109C was, additionally, a carrier of mutation F508del.

The variation found for exon 13a matched that of the positive control. However, and in order to corroborate that the change in electrophoretic profile was caused by the presence of mutation 2183 AA/G (figure 5), an amplification was performed with primers E13xR2 and E13xD2 [18] Mutation 2183AA/G leads to premature termination during protein synthesis at codon 38 of exon 13, and has been reported in Canada, Italy, Greece, Bulgaria, France, Germany, Turkey and Spain; it has been found in heterozygosis with mutations F508del, G542X, G1244E and 2789+5G→A and in all cases resulted in a severe phenotype [19]. This patient was a compound heterozygote for I507del (an exon 10 variation) and homozygotic for allele 7T from IVS8-6T. An association of mutation 2183AA/G with allele 7T has previously been reported for population studies in Spain and Turkey, excepting two patients homozygotic for allele 9T. A study of microsatellites

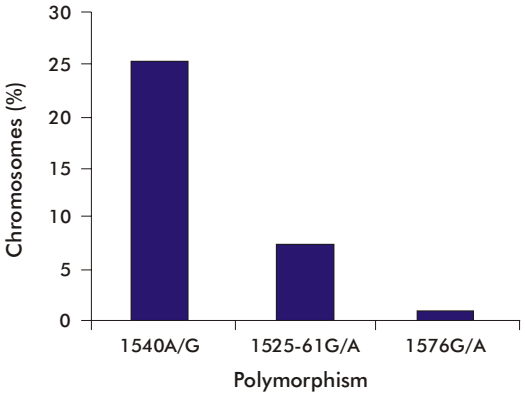

Figure 3. Distribution of polymorphisms 1540A/G, 1525-61 G-A and 1576 G/A. The x-axis represents the polymorphic identified variants, and the y-axis represents the percentage of the total analyzed chromosomes where each variant was found.

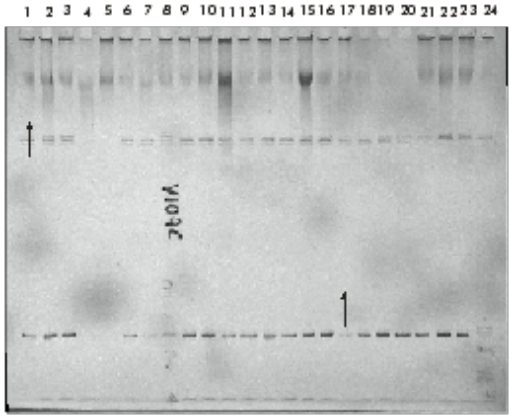

Figure 4. Results of the analysis by SSCP of exon 4, using 12% acrylamide gel. Lane 8 (from right to left) presents an anomalous pattern which does not match the positive control of the last lane. The remaining lanes contain normal electrophoretic profiles for exon 4 from CFTR.

IVS8CA and IVS17BTA revealed that this mutation was associated in the Spanish population to haplotype (16-30), and to haplotypes (16-31) and (16-32) in the Turkish population [20]. The Cuban patient was characterized as haplotype (16-31).

The analysis of the 23 samples from the FC Registry by DGGE identified changes in exons 17b, 11, 14a, 20 and 21; the analysis for exons 15, 14a, 20 and 21 is shown in figures 6 and 7.

In the case of exon 14a, a change was identified as corresponding to polymorphism 854T; likewise, a change corresponding to polymorphism 4002A/G was identified for exon 20. Both variants have previously been described for the Spanish population. No mutations in exon 15 were detected among the studied samples (figure 6).

During the analysis of exon 21 (figure 7), a variation was detected for sample number 3 which did not correspond to any of the known electrophoretic patterns found among the previously analyzed populations. This sample was analyzed by DNA

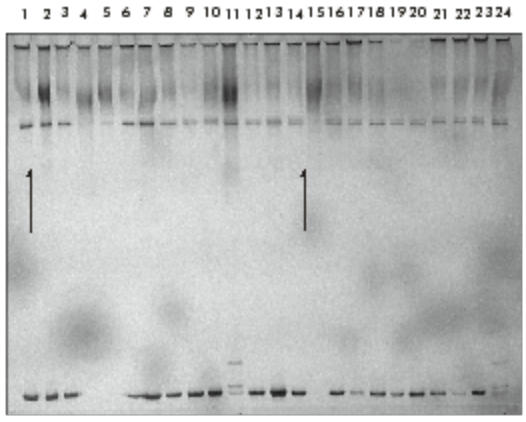

Figure 5. Results of the analysis by SSCP of exon 13a, using a 12% acrylamide gel. Lane 11 (from right to left) presents an anomalous electrophoretic profile which matches that of the positive control in the last lane. The remaining lanes contain normal patterns for exon 13a from CFTR.

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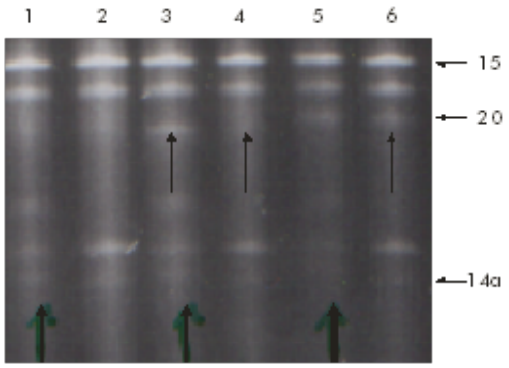

Figure 6. Results of the analysis of Multiplex B (Exon 15,14a and 20). Electrophoretic run in a denaturing acrylamide gel of the amplification products from exons 15, 20 and 14a of CFTR. There are changes for exon 20 in lanes 3, 5 and 6 and for exon 14a in lanes 1, 3 and 5. There are no changes in exon 15.

sequencing in order to determine whether the variation was a new polymorphism or a new mutation.

Figure 8 represents the electrophoretic profiles from exons 11, 14b and 17b. Variations found for exon 11 correspond to mutations R553X and G542X, and were further confirmed by amplification with primers EDM and I11R1, described by Morral *et al* [21], for the cases carrying mutation R553X or by the amplification-refractory mutation system (ARMS) [22] for the cases carrying G542X. Two patients were identified as R553X carriers, and one of them was characterized as a compound heterozygote with F508del. In the case of the G542X heterozygote, mutation 3272-26A→G was found in the remaining allele. No changes for exon 14b were detected among the studied samples.

The analysis of exon 17b, which used as a positive control a case with mutation 3272-26A→G, detected a sample with the same electrophoretic variation which was further confirmed by amplifying this region with the specific primers 17bxD1 and E17R1, reported by Amaral *et al* [23]. This mutation generates an mRNA carrying an additional nucleotide that results in

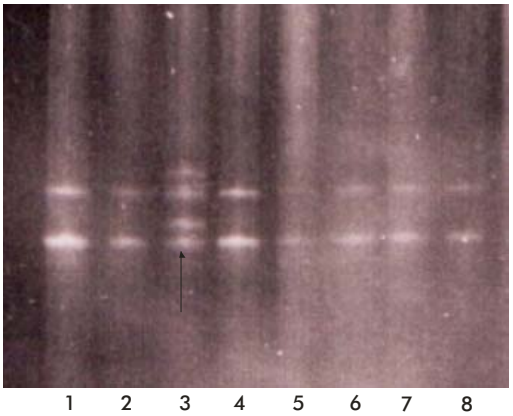

Figure 7. Analysis by DGGE of exon 21, using denaturing acrylamide gel electrophoresis of the amplification products from this region. A change in electrophoretic mobility can be seen in lane 3, corresponding to a CFTR exon 21 mutation or polymorphism.

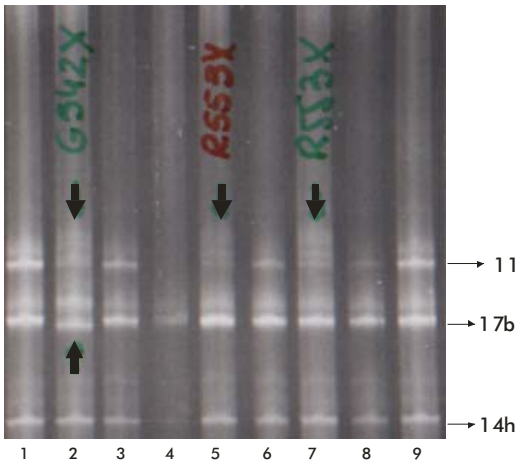

Figure 8. Multiplex A exon 11, 14b and 17b. Electrophoretic run in a denaturing acrylamide gel of the products of amplification from exons 11, 17b and 14b from CFTR. A change in electrophoretic mobility corresponding to mutation G542X can be observed in lane 2 at exon 11; likewise, lanes 5 and 7 have an electrophoretic pattern coinciding with mutation R553X in the same exon. Lane 2 at exon 17b also shows a change corresponding with mutation 3272-26G→A. No changes are observed for exon 14b.

premature transcriptional termination, and has been previously found in patients from France, Spain, Belgium, Greece, Germany and Portugal, often in heterozygosis with mutations F508del, G542X, N1303K and W846X, among others. In general these patients have severe clinical manifestations, excepting the 3272-26A>G/L206W and 3272-26A>G/P99L compound heterozygotes [23]. The carrier of this mutation in our sample had mutation G542X, corresponding with a severe clinical picture of CF.

Table 2 summarizes all the mutations detected either by SSCP or DGGE and the cases which could be fully characterized. A complete identification of the 2 mutant alleles was achieved for only 6 out of the 23 patients, in contrast with the identification rates described in the CF literature for DGGE and SSCP, which are often higher than 90%.

The diagnosis of cystic fibrosis is based on the presence of a multisystemic clinical symptomatology compatible with the disorder, together with two or more assays for electrolytes in sweat [24-30]. The clinical symptoms of this disease are usually highly heterogeneous, with multiple manifestations [24, 31, 32] of varying intensity and evolution, even between patients with the same genotype belonging to the same family. In many cases, a proper diagnosis is complicated due to the similarity of the respiratory clinical

Table 2. Mutations detected in 6 patients by SSCP or DGGE

Patients	Altered exons	Identified mutation
1	11 and 17a	G542X / 327226A→ G
2	10 and 11	F508del / R553X
3	10 and 4	F508del / Y109C
4	10 and 13a	I507del / 2183AA/G
5	10 and 14a	To be sequenced
6	10 and 21	F508del / To be sequenced

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symptoms with those caused by other affections, which are highly frequent in Cuba.

Conclusions

A proposal was presented by a group of researchers in the year 2000 for a revision of the diagnostic criteria for CF which would take into account the need to differentiate between cystic fibrosis and other disorders whose genetic basis were the presence of mutations in the CFTR gene [25]. This proposal eventually resulted in the presentation, in June 2000, of a new classification to the International Cystic Fibrosis Association at the WHO [33]. Based on that classification, the Cuban National Cystic Fibrosis Commission re-evaluated all the investigated cases, and after this study, the specialists concluded that of the 23 cases analyzed by SSCP and DGGE, only 6 were actually cystic fibrosis patients with confirmed mutations in both *cftr* alleles. This result corroborated the efficacy of the selected screening method and evidenced the existence of over-diagnosis for CF in our country.

Acknowledgements

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