THE ANALYSIS OF SOME *CFTR* GENE MUTATIONS IN A SMALL GROUP OF CF PATIENTS FROM SOUTHERN PART OF ROMANIA

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Abstract. Cystic fibrosis is the most common hereditary disease in European descendant populations, with prevalence depending on ethnic groups studied. In contrast to other European countries, there is little information regarding the frequency of CFTR mutations for the Southern part of Romania. The aim of this study was to test the presence of nine CFTR mutations in CF patients from the Southern part of Romania, using complementary analysis methods. We investigated a group of unrelated CF patients (n=19) and, when possible, their voluntary parents (n=15). We observed that the most frequently worldwide CF mutation, ΔF508, was present in 17 of our patients (89.5%) in homozygous (n=7) or heterozygous (n=10) condition and absent in 2 cases (10.5%). This mutation was also detected in ten parents, seven of them (100%) have homozygous children and three (37.5%) have heterozygous children for ΔF508 mutation. None of the G542X, S549N, G551D, R553X, R560T, S1255X, W1282X and N1303K mutations have been detected in the samples from patients or parents. Our results are partially similar with those reported in neighbouring countries where the ΔF508 is the most common mutation detected and the frequency of R560T, S549N, G551D and S1255X mutations is near zero. The enlargement of this study could give a better result regarding the spectrum of CFTR mutations in Romanian patients with CF.

Keywords: Cystic fibrosis, *CFTR* gene, ΔF508, mutations

INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive disorder, caused by mutations in the cystic fibrosis transmembrane conductance regulator gene (CFTR) on chromosome 7q31. The normal *CFTR* gene product is a chloride channel found in the apical membrane of epithelial cells, able to regulates other ion channels [1, 11, 22, 23]. A defective CFTR protein determines an abnormal chloride transport, elevated sweat chloride and the production of thick and sticky mucus. This mucus clogs in the lung, intestines, exocrine pancreas and reproductive systems. Recurrent and persistent pulmonary infections lead to respiratory failure which is the main cause of morbidity and mortality. Although elevated sweat chloride values sustain a clinical diagnosis of CF, the testing for disease-causing mutations is helpful for establish the definitive diagnostic.

More than 1600 sequence variants have been reported since the cloning of the *CFTR* gene [4, 13, 21]. Few of them are worldwide present, several are distributed only in an ethnic group, while the great majority are very rare, being considered regional or "private". The mutation ΔF508, consisting in the deletion of three nuclotides, is present at majority (~70%) of patients with CF [13]. The high prevalence of the CF in certain populations could be explained by recurrent mutations, the founder effect or by the heterozygote advantage [20]. The CF has the highest incidence in Caucasians and Ashkenazi Jews (1:2500 and 1:2270, respectively), while the lower are in Asian and African populations [10, 18]. The CFTR mutations in Romania was investigated especially for patients from the Western part of country [8, 19].

The aim of this study was to test the presence of nine CFTR mutations in CF patients from the Southern part of Romania using complementary analysis methods.

MATERIALS AND METHODS

Unrelated CF patients (n=19) and their parents (n=15) who lived in the Southern part of Romania were selected from Bucharest pediatric hospitals. The CF diagnosis was based on clinical data and was confirmed by the sweat test (cutoff, 60 mEq/L). Informed oral consent was obtained from patient's parents before selection for this study.

Genomic DNA was extracted from all EDTA-treated blood samples using a classical protocol and then was stored at -20^{o}C. We tested nine mutations located in CFTR exons 10, 11, 20 and 21 (Table 1) [12, 16]. The specific restriction endonucleases (5 Units per reaction) were used to digest the amplicons for three hrs in conditions recommended by manufacturers. In order to give an accurate and rapid molecular diagnosis for the most common CF mutation, ΔF508, we used two alternative analysis methods: PCR-mediated site-directed mutagenesis [9] and high resolution melting analysis (HRM). After amplification, the first method requires PAGE (20%, TBE 1x, 5V/cm) of restriction products and silver staining, while the second requires the denaturation of amplicons. The PCR mixture (25 µl) contained 1.2 µl of genomic DNA, 12.5 µl of SensiMix HRM, 1 µl of EvaGreen (Quantace), 250 nM of each primer and double distillated water. We used the same primers as for the PCR-RFLP method. The temperature cycling conditions were: 10 minutes at 95°C followed by 45 cycles of 95°C for 15 seconds, 54°C for 25 seconds, 72°C for 10 seconds. This protocol continues with one cycle of 120 seconds at 95°C and 20 seconds at 72°C and is followed by a melt step of 72–90°C (0.1°C increment, pausing for 2 seconds per step) in a Rotor-Gene 6000 with HRM (Corbett Research).

The study was in accordance with the Declaration of Helsinki.

Table 1. Summary presentations of methods used in this study for mutations detection

Nr. crt.	Mutation	Type of mutation	Exon	Frequency worldwide	Method (restriction endonuclease)	Size of normal fragment	Size of mutant fragment
1.	ΔF508	Deletion	exon 10	66.8% [7]	PCR-mediated site-directed mutagenesis (MnlI)	56+30 bp	83 bp
2.	G542X	Substitution	exon 11	2.6% [10]	PCR-mediated site-directed mutagenesis (MvaI)	171+100 bp	271 bp
3.	S549N	Substitution	exon 11	0.1% [2,17]	PCR-RFLP (DdeI)	13, 238+174 bp	13+412 bp
4.	G551D	Substitution	exon 11	1.5% [10]	PCR-RFLP (MboI)	425 bp	182+243 bp
5.	R553X	Substitution	exon 11	0.7% [10]	PCR-RFLP (Hinc II)	239+186 bp	425 bp
6.	R560T	Substitution	exon 11	0.2 % [21]	PCR-RFLP (TaiI)	425 bp	215+210 bp
7.	S1255X	Substitution	exon 20	0.2% [21]	PCR-RFLP (HindIII)	473 bp	261+212bp
8.	W1282X	Substitution	exon 20	1% [10]	PCR-RFLP (MnlI)	288 +185 bp	473 bp
9.	N1303K	Substitution	exon 21	1.6 [10]	PCR-mediated site-directed mutagenesis (MvaI)	266+24 bp	290 bp

RESULTS

The biological samples were screened for the presence of the nine CFTR mutations by PCR-RFLP methods. For the homozygous ΔF508 affected individuals, a single 83-bp fragment is revealed, and for the normal condition, two fragments of 56- and 30-bp are present. The ΔF508 in heterozygotes state is considered when 83-bp, 56- and 30-bp were identified. An additional band, representing the heteroduplex, may be also encountered. It represents an indicator of heterozygosity, but not a proof for a specific mutation (Fig. 1) [9]. The ΔF508 mutation was absent in 2 (10,5) patients and was identified in 17 patients (89.5%) in homozygous (n=7) or heterozygous (n=10) condition. This mutation was also detected in ten parents, seven of them (100%) have homozygous children and three (37.5%) have heterozygous children for the ΔF508 mutation. No other mutation has been detected in the patients or in the parents groups (Table 2).

We obtained by HRM analysis three patterns corresponding to the three conditions: homozygote normal, heterozygote ΔF508/N and homozygote ΔF508 (Fig. 2). This analysis confirms the results provided by the PCR-RFLP method.

Table 2. Type of CFTR mutations detected in the analyzed samples

Nr. Crt.	Mutation	Patient's genotype		Parent's genetic status	
		Homozygous	Heterozygous	Carrier	Non-carrier
1.	ΔF508	7	10	10	5
2.	G542X	0	0	0	0
3.	S549N	0	0	0	0
4.	G551D	0	0	0	0
5.	R553X	0	0	0	0
6.	R560T	0	0	0	0
7.	S1255X	0	0	0	0
8.	W1282X	0	0	0	0
9.	N1303K	0	0	0	0

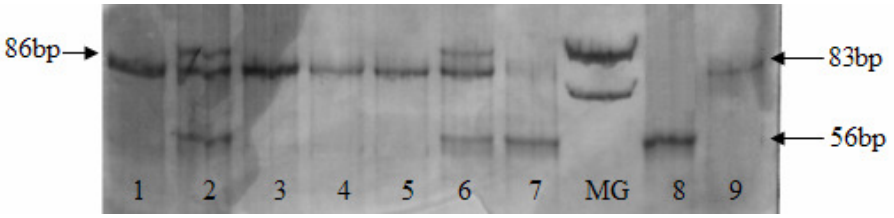

Figure 1. The result of a test for the ΔF508 mutation (PAGE 20%, silver staining). Lines 1, 3, 4, 5, 9 – genotype ΔF508/ ΔF508; Lines 2, 6 – genotype ΔF508/n; Lines 7 , 8 – normal genotype , MG – 100 bp DNA ladder (Fermentas)

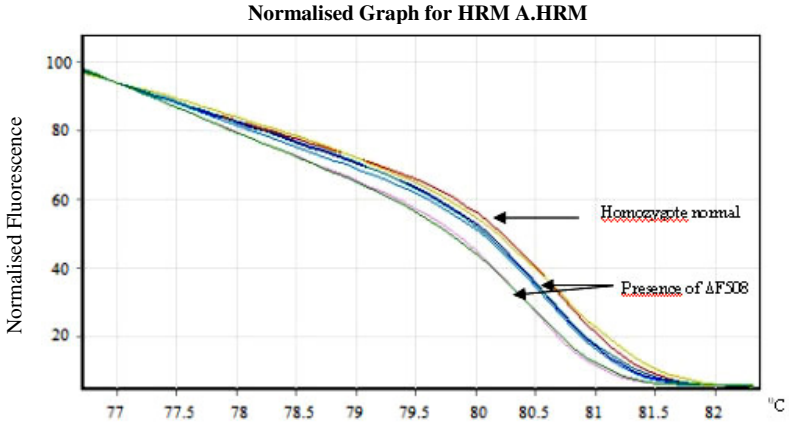

Figure 2. Analysis of DF508 mutation by HRM

DISCUSSIONS

In the majority of cases, the clinical diagnosis of CF is confirmed by elevated (>60 nmol/l) sweat chloride concentrations and, when possible, by screening the *CFTR* gene for mutations. However, because this gene has a high number of very rare mutations, the possibility to test all CFTR mutations for each CF patients is practically impossible. In the last time, the detection rate of mutations was improved in different research centers by different methods: heteroduplex analysis (HA), single-strand conformation polymorphism analysis (SSCP), denaturing gradient gel electrophoresis (DGGE) methods, two-dimensional gene scanning technique (TDGS) and HRM (High resolution melting) analysis [3, 14, 15], allele specific oligonucleotide (ASO), amplification refractory mutation system (ARMS), oligo-ligation assay. Different commercial kits also provided solutions for detection of the most common mutations. However, a panel of 25 of the most frequently mutations were recommended to be tested for CF molecular diagnosis. For the compound heterozygous patients, carrying one common and one very rare mutation, additional tests are required [5, 6].

In order to test the presence of some CFTR mutations, we tested blood samples from patients with CF (n=19) and 15 of their parents living in the Southern part of Romania. In this study we investigated the most frequently five mutations, represented by ΔF508, G542X, N1303K, G551D and W1282X. All of these mutations have a worldwide estimated frequency higher than 1% (Table 1). We have also analyzed other four mutations, S549N, R553X, R560T and S1255X, which have a frequency lower than 1%, but are located nearby some mutations analyzed in this study. Some studies which have analyzed the genotype-phenotype correlation, have been shown that the nine mutations investigated in our study are associated with a severe CF phenotype, being included in the 25 mutations panel recommended to be used for a population screening [25]. We also try to determine the feasibility of HRM analysis for the detection of CFTR mutations. In literature, HRM is estimated to be able to discriminate samples which present small mutations, like substitutions [24, 26]. By our experience, although HRM represent a fast method for detection of the common ΔF508 mutation, the results are difficult to be replicated. A possible explanation is represented by the protocol used for DNA extraction or by other technical problems.

The ΔF508 mutation, in homo- (n=7) or heterozygous (n=10) state, was observed in the majority of our patients (89.5%).

The absence of the eight mutations tested could be explained by the lot size and, partly, by the background of geographic area. Thus, the S549N, R560T and S1255X mutations were reported to have a null frequency in the majority of neighboring countries. It has been showed that in samples from Russia, Estonia, Lithuania, Ukraine, Hungary, Bulgaria, Turkey, Croatia, Macedonia and Greece the mutation G551D was identified with a frequency of 1,1% only in Croatia, while the mutations S549N, R560T and S1255X were not present [2]. The data obtained for Romanian CF patients were also presented in another two studies: one for the Western part of country and the other for patients enrolled from different medical centers [8, 19]. The highest mutation frequency was attributed to ΔF508 in both of these studies, as well as in our investigation. The G551D, S549N, R553X, R560T, S1255X mutations assessed but not detected in the present investigation have not been included in any of the two already mentioned studies.

Because the remaining mutations were not identified in a previous study on the Romanian population, it is possible that their frequency to be as smaller as those reported for general European population.

The molecular analysis used in this study could be applied for testing other mutations in *CFTR* gene. The investigation of the whole *CFTR* gene in patients from the entire Romania will improve our knowledge about the type and the frequency of the CFTR mutations.

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