GENETIC ANALYSIS OF CFTR MUTATIONS IN CYSTIC FIBROSIS PATIENTS FROM ROMANIA

Liviu Tamas¹, Ioan Popa², Liviu Pop², Andrei Anghel¹, Zagorca Popa³, Catalin Marian¹

Abstract

Objective: Cystic fibrosis is the most common autosomal recessive disease in Caucasian populations. The aim of this study was to improve the number of cystic fibrosis mutations detected in patients from the National Center of Cystic Fibrosis from Timisoara and to establish a solid method of genetic analysis for cystic fibrosis mutations in Timisoara. Material and methods: The study included a retrospective part, which consisted of analyzing the genetic tests results for 79 patients from the National Center of Cystic Fibrosis from Timisoara, already investigated in collaboration with Royal Manchester Children’s Hospital - Genetic Unit (UK), and an original, prospective part, in which we selected 17 patients in evidence at the Center for genetic analysis, based on clinical findings and sweat test. 29 mutations were investigated and the detection was performed using a Elucigene CF29 kit, which detects point mutations or small deletions in deoxyribonucleic acid (DNA) using a method based on ARMS allele specific amplification technology. DNA was extracted from lymphocytes from peripheral blood samples. genomic DNA was amplified by PCR and the PCR products were visualized on a UV transilluminator after electrophoresis on agarose gel and staining with ethidium bromide. Results: We identified 18 mutated alleles from a total number of 34 alleles and three mutations: ΔF508, G542X and I148T. The most frequent mutation in Western and Central Europe, ΔF508 (70%) has a lower frequency in Romania (47.92%). There are still many mutations that remain unidentified (34 %) in urma investigării numai a mutațiilor uzuale. Numărul mare de mutații și polimorfisme identificate până în prezent (25) demonstrează heterogenitatea genetică a populatiei României. Cuvinte cheie: fibroză chistică, copii, mutație ΔF508

Introduction

Cystic fibrosis is the most common autosomal recessive disease in Caucasian populations, having a frequency of 1 in 2000 - 2500 live births and a carrier frequency of 1 in 25 - 30.² It is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene on chromosome 7, which codes a membrane-associated protein of 1480 amino acid residues that forms a cAMP-regulated and ATP-gated chloride channel.¹³

The CFTR protein, which is a low conductance chloride channel localized at the apical membrane of epithelial cells, belongs to the large superfamily of ATP-binding cassette (ABC) transporters.⁸-¹⁰

It is composed of two symmetrical halves, each possessing a membrane-spanning domain (MSD) and a nucleotide-binding domain (NBD, or the ABC
The regulatory (R) domain is the link between the two halves and the phosphorylation of the regulatory domain regulates channel activity.10,11 Patients with cystic fibrosis have abnormal chloride conduction across the apical membrane of epithelial cells, causing inspissated secretions in the airways, pancreas, intestines, and vas deferens.5 The main clinical symptomatology of the disease consists of:

a. lung disease with the following pulmonary symptomatology: bronchiectasis, atelectasis, hyperinflation, airway obstruction, recurrent/chronic pneumonia, Pseudomonas sp. chronic infections.

b. gastrointestinal and nutritional abnormalities: pancreatic insufficiency, chronic diarrhea, failure to thrive, meconium ileus and rectal prolapse.

c. High values for the sweat test (high chloride concentration in sweat).1,5,12-16

The aim of this study was to improve the number of detected CFTR mutations in cystic fibrosis patients from Romania, to calculate a new frequency for CFTR mutations and to establish a solid method of genetic analysis for cystic fibrosis mutations in Timisoara.

MATERIALS AND METHODS

The patients were selected over a period of two years based on typical clinical findings and sweat test values. The selection was made from patients who are in evidence at the National Center of Cystic Fibrosis from Timisoara.

We analyzed 34 chromosomes from 17 patients which presented clinical manifestations of the disease and high values for the sweat test. We also investigated patients who presented normal or borderline values for the sweat test, but had suggestive clinical manifestations.1,2

Genetic analysis was performed on samples of whole blood (EDTA). The samples of blood were either immediately analyzed or frozen at −200°C for later analysis.17


The quantity and quality of DNA extracted by the three methods was similar and further analysis of extracted DNA produced optimal results. DNA concentration was verified by electrophoresis on agarose gel (0.4%) with a DNA weight marker (10 ng/μl), obtaining results in the interval 10-30 ng/μl, optimal concentrations for PCR amplification.

For one sample, genomic DNA was amplified by PCR according with the Elucigene™ CF29 kit protocol using a 25 μl reaction mixture for each of the four primers mix: 5 μl DNA and 20 μl of reaction mixture - 5,5 μl enzyme(AmpliTaq Gold) dilution (with sterile deionised water, dilution buffer and loading dye) + 16,5 μl primers mix(TA, TB, TC or TD), from which we used 20 μl for mixing with DNA in 4 thin-walled PCR vials. A negative DNA control was included in each PCR run.18

PCR amplification was carried out in a Touch Gene Gradient Thermocycler (Techne, Massachusetts, USA) using the amplification program from Elucigene™ CF29 kit protocol (activation of the AmpliTaq Gold at 94°C for 20 minutes linked to an amplification cycling program of 30 seconds at 94°C - denaturation, 2 minutes at 58°C - annealing and 1 minute at 72°C - extension, for 35 cycles. This was linked to a 20-minute time-delay file at 72°C - extension on the final cycle).18

The next step was the electrophoresis of the PCR products (25 μl) on a 3% agarose (NuSieve 3:1, Cambrex BioScience) gel using tris-borate with ethidium bromide (TBE/EtBr) as running buffer in a Sub-Cell GT DNA Electrophoresis system (Bio-Rad). A 50 Base-Pair Ladder (Amersham-Pharmacia Biotech) at 1.5 mg/15 ml was prepared in the Loading Dye supplied (80 ml distilled water/10 ml Loading Dye /10 ml 50 Base-Pair Ladder). 25 ml of this dilution was loaded on the gel and run adjacent to samples as a molecular weight marker.
Electrophoresis was carried out at 5 V/cm between electrodes (for a gel 15 x 25 cm and a distance of 30 cm between electrodes we used 150 V) until the dye front had migrated 5 – 10 cm from the loading wells towards the anode (1.5 to 2 hours).

After electrophoresis the gels were placed on a UV transilluminator at 260 nm, then visualized and photographed with a Polaroid® Instant Camera (Cole-Parmer) or a digital camera.

RESULTS

Genetic analysis led to the detection of 3 different mutations: ΔF508, G542X and I148T.

The most prevalent mutation, ΔF508, was found in 15/34 CF chromosomes (Figures 1, 2), with the second most common allele being the G542X (2/34) (Fig. 2) and I148T (1/34). (Fig. 3) 16 (47.06%) alleles remained unidentified. (Table 1)

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Number of alleles</th>
<th>%</th>
<th>Cumulative</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔF508</td>
<td>15</td>
<td>44.11</td>
<td>44.11</td>
</tr>
<tr>
<td>G542X</td>
<td>2</td>
<td>5.88</td>
<td>49.99</td>
</tr>
<tr>
<td>I148T</td>
<td>1</td>
<td>2.94</td>
<td>52.93</td>
</tr>
<tr>
<td>Unknown</td>
<td>16</td>
<td>47.06</td>
<td>100</td>
</tr>
</tbody>
</table>

In Table 2 we show the different CFTR genotypes. The most frequent genotype was ΔF508/ΔF508 (5/17). Other genotypes identified were: ΔF508/unknown observed in 4 cases, AF508/non-ΔF508 found in 1 patient, non-ΔF508/unknown in 2 and unknown/unknown, in 5. We named as unknown the alleles with mutations that couldn’t be detected by the Elucigene kit, due to its limitations (it can detect only 29 mutations) and non-ΔF508 – the alleles with mutations, other than ΔF508, that were identified by the Elucigene kit (G542X, I148T). Of the 17 patients tested 6 had both mutations detected, 6 only one mutation identified, and in 5 both mutations remained unknown.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Number</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔF508/ΔF508</td>
<td>5</td>
<td>29.41</td>
</tr>
<tr>
<td>ΔF508/G542X</td>
<td>1</td>
<td>5.88</td>
</tr>
<tr>
<td>ΔF508/Unknown</td>
<td>4</td>
<td>23.53</td>
</tr>
<tr>
<td>G542X/Unknown</td>
<td>1</td>
<td>5.88</td>
</tr>
<tr>
<td>I148T/Unknown</td>
<td>1</td>
<td>5.88</td>
</tr>
<tr>
<td>Unknown/Unknown</td>
<td>5</td>
<td>29.41</td>
</tr>
</tbody>
</table>

DISCUSSIONS

To date more than 1400 mutations and polymorphisms have been identified in the CFTR gene.21,22 The most frequent mutation, ΔF508, accounts for approximately 70% of the CF chromosomes in northern and central European countries (and in the world), but the frequencies and types of mutations in different populations vary considerably depending on the ethnic and geographical origin of the population tested.23

Wide variations in disease manifestations are observed among affected CF patients and a multitude of disease-causing mutations have been found in the CFTR gene.

In Romania, previous studies on patients from the National Center of Cystic Fibrosis from Timisoara, consisted of genetic analysis for CFTR mutations and the following mutations and polymorphisms

The molecular diagnostic for identification of CFTR gene mutations at patients from Timisoara started to be performed since 1990, right after the discovery of CFTR gene in 1989 by Lap-Che Tsui and J.R.R Riordan. In 15 years, by collaboration with Royal Manchester Children’s Hospital – Genetic Unit (UK), blood samples from 79 patients (158 alleles) were analyzed and 25 mutations and polymorphisms were detected, including two new mutations - R735K and 1717-2 (A>G). 77 alleles were identified as ΔF508 (48.73%), non - ΔF508 – 30 alleles (18.98%) and 51 remained unidentified (unknown) - 32.27%. (Table 3)

Table 3. Frequencies of CFTR mutations observed in cystic fibrosis patients from Romania (total number of alleles = 158).

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Number of alleles</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔF508</td>
<td>77</td>
<td>48.73</td>
</tr>
<tr>
<td>non - ΔF508</td>
<td>30</td>
<td>18.98</td>
</tr>
<tr>
<td>Unknown</td>
<td>51</td>
<td>32.27</td>
</tr>
</tbody>
</table>

The 79 genotypes analyzed were distributed as follows: 26 genotypes were ΔF508/ΔF508, 8 - ΔF508/X, 16 - ΔF508/non-ΔF508, 4-non-ΔF508/non-ΔF508, 5-non-ΔF508/X and 20 unknown (X/X).24-29 (Table 4)

Table 4. Frequencies of the identified CFTR genotypes in cystic fibrosis patients from Romania (total number = 79).

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Number</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔF508/ΔF508</td>
<td>26</td>
<td>32.31</td>
</tr>
<tr>
<td>ΔF508/non-ΔF508</td>
<td>16</td>
<td>20.24</td>
</tr>
<tr>
<td>ΔF508/Unknown</td>
<td>8</td>
<td>10.12</td>
</tr>
<tr>
<td>Non-ΔF508/non-ΔF508</td>
<td>4</td>
<td>5.06</td>
</tr>
<tr>
<td>Non-ΔF508/Unknown</td>
<td>5</td>
<td>6.33</td>
</tr>
<tr>
<td>Unknown/Unknown</td>
<td>20</td>
<td>25.32</td>
</tr>
</tbody>
</table>

The actual prospective part of the study consisted of the genetic analysis on 17 new patients (the analyses were realized by the Division of Molecular Biology, Biochemistry Department from Victor Babes University of Medicine and Pharmacy, Timisoara). The new, original results (17 patients, 34 alleles) were combined with the previous results from the retrospective part of the study (79 patients, 158 alleles) and we obtained the following distributions, presented in Tables 5 and 6 (96 patients, 192 alleles).

Table 5. New frequencies of CFTR mutations observed in cystic fibrosis patients from Romania (total number of alleles = 192).

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Number of alleles</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔF508</td>
<td>92</td>
<td>47.92</td>
</tr>
<tr>
<td>Non-ΔF508</td>
<td>33</td>
<td>17.19</td>
</tr>
<tr>
<td>Unknown</td>
<td>67</td>
<td>34.89</td>
</tr>
</tbody>
</table>

Table 6. New frequencies of the identified CFTR genotypes in cystic fibrosis patients from Romania (total number = 96).

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Number</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔF508/ΔF508</td>
<td>31</td>
<td>32.29</td>
</tr>
<tr>
<td>ΔF508/non-ΔF508</td>
<td>17</td>
<td>17.70</td>
</tr>
<tr>
<td>ΔF508/Unknown</td>
<td>12</td>
<td>12.50</td>
</tr>
<tr>
<td>Non-ΔF508/non-ΔF508</td>
<td>4</td>
<td>4.16</td>
</tr>
<tr>
<td>Non-ΔF508/Unknown</td>
<td>7</td>
<td>7.29</td>
</tr>
<tr>
<td>Unknown/Unknown</td>
<td>25</td>
<td>26.04</td>
</tr>
</tbody>
</table>

Also, it is noteworthy that the number of alleles with ΔF508 mutation (15) is similar with the number of alleles with unknown mutations (16), which supports the idea that the Romanian population is an heterogeneous one regarding CFTR mutations. (Table 1)

In northern and central Europe the frequency of ΔF508 is approximately 70%, but in Romania the frequency of ΔF508 remains low (47.92%), the non - ΔF508 alleles account for 17.19 % and the unidentified mutations still have a high frequency (34.89%). The genetic heterogeneity of Romanian population for the CFTR gene (25 mutations detected) and the low frequency of ΔF508 are similar with the situation from other eastern or southern European countries (Hungary ΔF508 - 54.9%, Greece - ΔF508 - 52.9%, Bulgaria - ΔF508 - 63.6%, Macedonia - ΔF508 - 54.3%). It is interesting to observe that a low frequency of ΔF508 appears also in other Latin European countries like Italy - 50.9%, Portugal - 44.7% and Spain - 52.7%.

Also like Romania, Spain, Bulgaria, Greece, and Turkey have some of the most diverse mutational arrays in Europe, on average approximately 25 mutations. This is most likely due to their geographic situation, serving as historic gateways into Europe from the Middle East, Africa, and associated waterways.

Countries from central, northern, western, and northeastern Europe show a large degree of homogeneity among CFTR mutations (Austria, Belarus, Belgium, Denmark, Estonia, Finland, France, Germany, Lithuania, the Netherlands, Norway,
Poland, Russia, Sweden, Switzerland, (and the Ukraine) and a reduced mutational arrays (approximately 10 mutations).

The second most frequent mutation in Romania is G542X (4 alleles/192 alleles), which is also second in Europe and in the world. G542X is most common in the Mediterranean regions of Europe and Africa. G542X has been implicated as a mutation that was introduced into the Mediterranean region by the migration of Phoenicians.28

Across Europe, G542X is found in significantly higher proportions in countries, and in regions of countries, which border the Mediterranean Sea. G542X has its highest rates of prevalence in the north of Africa and in the south of Spain (South Spain, 14.4%; Tunisia, 8.9%). Interestingly enough, these two regions correspond to two major ancient Phoenician cities, Carthagène and Carthage, respectively.28

The genetic results of the introduction of this mutation into such a busy trade and trafficking center as the Mediterranean can still be observed today. When one studies CF chromosomes in those populations of the New World that were heavily influenced by such countries as Spain and Italy (countries from South and Central America), the G542X mutation frequencies are virtually identical.28

The mutation N1303K (which has also a higher frequency comparing with the others mutations with the exception of ΔF508) is another CFTR allele that has a similar distribution patterns as G542X, and may also have been introduced via the Mediterranean route.

W1282X is a mutation of single origin that has historically been associated with the Ashkenazi Jews. This mutations frequency has been amplified to almost double that of the ΔF508 mutation in this distinct population.28

In Romania W128, N1303K are more frequent then the others mutations (2 alleles each from 192 alleles) and there also several mutations which have the same frequency: CFTR dele 2,3 (21kb)37, 2183 AA >G, 621 +1 G >T and I148T. The I148T mutation has a similar distribution patterns as G542X, and has a higher frequency in the French-Canadian population.28

In Romania W128, N1303K are more frequent then the others mutations (2 alleles each from 192 alleles) and there also several mutations which have the same frequency: CFTR dele 2,3 (21kb)37, 2183 AA >G, 621 +1 G >T and I148T. The I148T mutation has a higher frequency in the French-Canadian population (9.1%), while in other populations it is a less frequent mutation (< 1%).21 The rest of the identified mutations are each represented by a single allele.

There are also two mutations which have been identified for the first time in Romania: R735K and 1717-2 (A>G). 24 That is why the use of commercial kits, like ElucigeneTM CF29 is not enough for detecting a significant percent (> 90%) of the mutations that appear in patients with cystic fibrosis. Therefore, for further improvement of genetic diagnostic we need to apply different methods like DNA direct sequencing or DGGE (Denaturing Gradient Gel Electrophoresis).48-56

The actual study shows the possibility of molecular diagnosis for the majority of cystic fibrosis patients and can offer for some cases the option of prenatal diagnosis.

CONCLUSIONS

1. The low frequency of ΔF508 (48%) in Romania and the great number of mutations and polymorphisms identified up to date reflects the genetic heterogeneity of Romanian population.25

2. The great number of unidentified mutations (34%) imposes the continuation of the study using multiple methods of mutation detection in order to detect them.

3. We succeeded to establish locally, in Timisoara, a solid method of molecular diagnostic for cystic fibrosis patients.

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