Prenatal Genetic Diagnosis by ARMS-PCR Method in Couples of CFTR Mutations Carriers

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Abstract:
The aim of this study was to detect CFTR mutations in genomic DNA isolated from amniotic fluid collected by amniocentesis and to establish if the fetus is just a carrier for one CFTR mutation or both alleles are affected and the fetus has cystic fibrosis.

Based on family history, 3 couples were selected for prenatal genetic diagnosis. The couples had previously children with cystic fibrosis, registered at the National Center of Cystic Fibrosis in Timisoara, who had been genetically tested and had both mutations identified, or had deceased children who had been diagnosed with cystic fibrosis but without molecular diagnosis. Both parents were also tested for CFTR mutations. Fetal genomic DNA was isolated from amniotic fluid collected by transabdominal amniocentesis in the 16th week of pregnancy. Genomic DNA from the parents was isolated from venous blood collected on EDTA. The genetic analysis for CFTR mutations was performed using the Elucigene CF29 kit and electrophoresis of ARMS-PCR products in agarose gel.

After analyzing the electrophoresis results, we identified 1 heterozygous genotype (ΔF508/N, G542X/N), one normal genotype and one compound heterozygote (621+1G>T/ΔF508).

Prenatal diagnosis can be performed by ARMS-PCR using Elucigene CF29 kit only in cases where the detected genotype has at least one allele with ΔF508 mutation or is a compound heterozygote for the other mutations detected by the kit, because this method doesn’t allow the detection of homozygous genotypes for mutations other than ΔF508. The results obtained by prenatal diagnosis are essential for a complete and successful genetic counseling.

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Keywords: cystic fibrosis, prenatal diagnosis, amniocentesis, CFTR mutations

Introduction

Mucoviscidosis is the most common genetic autosomal recessive disease in Caucasian populations, with an incidence of 1/2200 – 2500 live birth and a carrier frequency of 1 in 25 – 30 persons. The disease is caused by mutations of the CFTR gene (cystic fibrosis transductance conductance regulator), which is located on chromosome 7 in locus q31.2. The CFTR gene is a large gene (250 kbp) with 27 exons, which produces a 6.5 kbp RNA messenger, the final product being a transmembrane protein with 1480 aminoacids which form an AMPc-regulated ATP-dependent chloride channel1,2.

The CFTR protein, a low conductance chloride channel, is located in the apical membrane of epithelial cells found in pancreatic, biliary and intestinal ducts, in the tracheobronchial tubes, uniriniferous tubules, genital
ducts and sweat glands. It belongs to the larger family of carrier proteins – ABC carriers (ATP Binding Cassette transporters).

The protein is made up of two symmetrical halves, each consisting of a membrane-spanning domain (MSD) and a nucleotide-binding domain (NBD or the ABC domain). The regulating domain (R) represents the link between the two halves, and its phosphorylation regulates protein channel-activity.

Patients with mucoviscidosis suffer from a chloride ion conductance dysfunction, the protein being absent from the apical membrane of epithelial cells or, if present, it is non-functional, lacking the ability to carry chloride ions from the intracellular area to outside the cell, which induces intracellular water retention also resulting in dehydration of secretions of the mucus, which thus becomes viscous. In the sweat glands, chloride ion circulation takes place in a reverse direction which, in the case of mucoviscidosis, causes the accumulation of chloride ions outside the cell which in combination with sodium ions, that are mostly extracellular, give sweat its salty taste, characteristic for mucoviscidosis.

The clinical picture of the disease may include the following types of symptoms:

» pulmonary involvement caused by bronchial obstruction due to mucous build-up: bronchiectasis, atelectasis, hyperinflation, respiratory tract obstruction, recurrent/chronic pneumonia, chronic pulmonary infections with Pseudomonas sp;

» gastrointestinal and nutritional involvement: pancreatic failure, chronic diarrhea, growth failure, meconial ileus and rectal prolapse;

» high values of sweat test (high level concentrations of chloride ions in the sweat).

The clinical picture of mucoviscidosis patients may vary, depending on the existing CFTR mutations, so that the disease may have severe forms, rapidly evolving to patient mortality, or milder forms without pulmonary or pancreatic involvement. On average, patients that receive proper treatment since birth, survive up to the age of 20 – 30 years.

As most forms of the disease are severe ones and are associated with both pulmonary and pancreatic damage, eventually resulting in patient mortality, couples at risk for having children with mucoviscidosis should benefit from proper genetic counseling including prenatal genetic diagnosis, in such a way that these couples should enjoy the possibility of having healthy children and have access to all the information necessary to make an informed decision regarding the pregnancy.

Prenatal genetic diagnosis involves sampling of the amniotic fluid by amniocentesis carried out between the 15 and 16th week of pregnancy. Amniocentesis is an invasive method, but the risk of fetal injury is low (< 0.5 per cent).

**Material and methods**

Prenatal diagnosis was carried out in three couples who were carriers for CFTR mutations and had children with mucoviscidosis (registered in the database of the National Centre for Mucoviscidosis, Timisoara), that were genetically tested and had both mutations identified, or that already had deceased children clinically diagnosed with mucoviscidosis, but without a molecular diagnosis. Genetic testing was carried out for each parent, regardless of their family history or previous genetic tests.

Tests were carried out on venous blood samples collected on EDTA from both parents, and on amniotic fluid samples collected by transabdominal amniocentesis from the mother in the 15th – 16th week of pregnancy. 15 – 16 ml of amniotic fluid were collected (1 ml for each week of pregnancy); the first 5 – 6 ml were not used, only the remaining 10 ml, in order to avoid contamination of the amniotic fluid with blood or maternal cells. The amniotic fluid was also visually inspected after sampling to detect any possible traces of blood. Normal amniotic fluid is light yellow. DNA was extracted immediately after sampling in order to obtain the best results. Taking into consideration the low cellularity of the amniotic fluid, before DNA extraction, the samples of amniotic fluid were subjected to a mild centrifugation (1,500 – 2,000 rpm), the resulting supernatant (approximately 9 ml) was removed, the remaining sedimented fetal cells being resuspended in about 1 ml remaining fluid. Fetal cells resuspension was done by gently shaking the sample. These procedures aimed at concentrating fetal cells in the amniotic fluid and increasing the DNA quantity obtained following extraction. The blood samples taken from the parents were either immediately processed or preserved at -20°C for later analysis.
For detection of CFTR mutations, the authors used the Elucigene CF29 kit (Tepnel Diagnostics, UK), which can identify 29 mutations considered to be the most common among Caucasian populations: ΔF508, G542X, N1303K, 621 + 1G>T, 1717-1G>A, W128X, 3849 + 10kbC>T, R553X, G551D, R117H, R1162X, R334W, A455E, 2183AA>G, 3659delC, 1078delT, ΔI507, R347P, S1251N, E60X, D1152H, 3120 + 1G>A, 2789 + 5G>A, 1898 + 1G>A, 711 + 1G>T, G85E, 2184delA, I148T and R560T. At the same time, Elucigene CF29 can identify the normal allelic variant for the locus characteristic for the ΔF508 mutation, which is the most common CFTR mutation (approximately 70% for Western and Central Europe populations and the USA), so that one can differentiate between ΔF508 heterozygots (carriers of the mutation) and ΔF508 homozygots (in which both alleles are affected, being thus ill). For the rest of the mutations, differentiation between heterozygots and homozygots is not possible, but their low incidence seldom induces the occurrence of homozygots. The method used by Elucigene CF29 is based on allele specific amplification technology through ARMS-PCR (amplification refractory mutation system), which makes possible the identification of punctiform mutations or small deletions in the DNA molecule. The principle of ARMS-PCR technique is based on the fact that primers (oligonucleotides), which have a nucleotide at the 3’ end of the molecule that is not complementary with the corresponding nucleotide in the attachment area to the amplification sequence, will not be able to initialize DNA synthesis, thus blocking the amplification reaction. The selection of proper primers allows mutant or normal DNA sequences to be amplified and then detected. The Elucigene CF29 kit used primers designed in such a way as to make possible the amplification reaction only in those cases where there are mutating sequences, and not when the DNA sequence is normal except for the locus corresponding to the ΔF508 mutation, in which case both the mutating sequence and the normal one can be amplified. 

Genomic DNA was extracted from the lymphocytes found in the venous blood collected on EDTA, according to the protocol recommended by the producers of Elucigene CF29 (extraction with NH₄Cl, NaCl, EDTA, NaOH and Tris-base/HCl) and using an alternative method with a commercial kit Qiagen QIAmp DNA Blood Mini for DNA extraction from most blood samples and from all the samples of amniotic fluid. For the extraction of genomic DNA from the amniotic fluid, the extraction protocol (Qiagen) used for blood samples was slightly modified and adapted to the possibilities offered by the producers to enhance the concentration of extracted DNA. Thus, the volume of amniotic fluid was 500 μl, and the final elution time was extended to up to 5 minutes, the volume of the buffer solution being reduced to 150 μl. DNA concentration was quantified by electrophoresis on agarose gel (0.4 %), the samples migrating in parallel with a DNA concentration marker (DNA weight marker, 20 ng/μl). The results were situated in the 10 – 20 ng/μl interval for the blood samples and in the 1 – 2 ng/μl interval for the amniotic fluid samples, in both cases the concentrations being optimal for the amplification reaction to be effective.

The extracted genomic DNA was amplified following the amplification program from Elucigene CF29 work protocol. A mixture of reactants was obtained, containing 1.5 μl DNA polymerase (AmpliTaq Gold), 8.5 μl sterile deionized water, 2.5 μl buffer solution for dilution and 12.5 μl staining solution. The resulting mixture was divided into four equal parts of 5.5 μl in four sterile 0.5 ml Eppendorf tubes, each tube containing 16.5 μl primer mixture (TA, TB, TC, TD). Subsequently, 20 μl were taken from each tube which were then introduced into a thin 0.2 ml PCR tube followed by 5 μl of the extracted genomic DNA. A DNA negative control was included in each set of PCR samples.

PCR amplification was conducted in a Touch Gene Gradient Thermocycler apparatus (Technne, Massachusetts, USA), using the following amplification program: DNA AmpliTaq Gold polymerase activation at 94°C for 20 minutes, followed by 35 cycles consisting of: denaturation stage at 94°C for 30 seconds, primer attachment stage at 58°C for 2 minutes and extension at 72°C for 1 minute. At the end of the amplification program, the extension stage of the last cycle, at 72°C, was programmed to last 20 minutes.

**PCR product electrophoresis**

The migration of PCR products obtained by amplification was carried out on agarose gel 3 % (NuSieve 3:1; Cambrex BioScience), which was mixed during preparation with ethidium bromide (20 μl...
ethidium bromide solution to 200 ml gel). The migration buffer solution used was TBE (Tris-Borat-EDTA), and migration took place in a Sub-Cell GT DNA Electrophoresis system (Bio-Rad) apparatus. As marker of the size of the fragments obtained by PCR reaction, we used the 50 Base-Pair Ladder (Amersham-Pharmacia Biotech) with a 1.5 μl/15 μl concentration, from which a dilution was made following the producers’ recommendations (80 μl sterile deionized water/10 μl staining solution/10 μl Ladder 50 pb).

In each well of agarose gel we introduced 20 μl of the PCR products, and adjacent to these we loaded 20 μl marker dilution for the size of the fragments. The migration of PCR products took place at 4 – 5 V/cm calculated to the distance between the electrodes (for a gel of 6 x 7 cm and a distance of 20 cm between the electrodes, we used an electric potential of up to 80 V), until the dye front had migrated 5 cm from the loading wells towards the anode (1.5 to 2 hours).

Once electrophoresis was finished, the gels were visualized with an UV transiluminator at 260 nm and photographed with a Canon A430 digital camera with filters adapted to the corresponding wavelength of ethidium bromide light emission15,17.

Results and discussion

After photographing the gels, results were interpreted according to the diagram shown in Figure 1.

The first investigated couple among the visualized electrophoresis bands, showed the presence of a fragment of 279 pb (Fig. 2) which, according to the interpretation diagram, corresponds to the G542X mutation, thus the fetus being the carrier of this mutation (heterozygote). For the locus of the ΔF508 mutation, the normal sequence was found, the mutant sequence characteristic for the ΔF508 mutation being absent. No other electrophoresis bands were found to indicate the existence of other mutations, with the

**Fig. 1. Interpretation of the results obtained by electrophoresis of PCR products**

In each well of agarose gel we introduced 20 μl of the PCR products, and adjacent to these we loaded 20 μl marker dilution for the size of the fragments.

The migration of PCR products took place at 4 – 5 V/cm calculated to the distance between the electrodes (for a gel of 6 x 7 cm and a distance of 20 cm between the electrodes, we used an electric potential of up to 80 V), until the dye front had migrated 5 cm from the loading wells towards the anode (1.5 to 2 hours).
exception of normal control bands. The couple had already had a child with mucoviscidosis, complete form, with pulmonary and pancreatic involvement, who was genetically tested and showed the presence of G542X/ΔF508 genotype (compound heterozygote). Both the ΔF508 mutation and the G542X mutation are severe mutations that induce the occurrence of complete forms of the disease. Genetic testing of the parents showed that the mother was a carrier for ΔF508 (genotype ΔF508/N), and the father was a carrier of the G542X mutation (genotype G542X/N). As for the disease to become manifest requires the existence of at least two mutant alleles, the fetus in our case was considered to be only carrier of a CFTR mutation, and was going to be clinically healthy. The couple was told the information concerning the fetus’ health condition, and the recommendation made during genetic counseling was to continue the pregnancy.

The prenatal genetic diagnosis of the second couple showed a normal genotype, the ΔF508 mutation being absent (Fig. 3), the only noticeable thing being the
electrophoresis band characteristic for the normal sequence for the ΔF508 locus (ΔF508 N, 160 pb). The parents were previously tested, the mother being a carrier for G542X, and the father being a carrier for ΔF508. The couple had in their history a deceased child with the clinical diagnosis of mucoviscidosis, but who couldn’t be genetically tested. Following the result of prenatal diagnosis, the recommendation was to continue the pregnancy, the fetus having none of the mutations of the parents.

The last investigated couple had already had a child who had died in its first month, clinically diagnosed with mucoviscidosis, but who hadn’t been genetically tested for the confirmation of the molecular diagnosis. The parents were genetically tested and the results showed that the mother was a carrier of the 621+1 G>T mutation, and the father was a carrier of the ΔF508 mutation. Prenatal diagnosis showed that the fetus was a compound heterozygote for the two mutations (genotype ΔF508/621+1G>T), a condition that confirms the diagnosis of mucoviscidosis, both alleles being affected (Fig. 4). The parents were informed of the result, receiving full information on the child’s chances of survival with early proper treatment. The couple decided to terminate the pregnancy.

Conclusions

The ARMS-PCR method used by Elucigene CF29 offers the possibility of a fast and accurate prenatal diagnosis, with a good sampling technique for the amniotic fluid in order to prevent its contamination with blood or cells from the mother, and a very good technique for blood DNA extraction.

The technique is applicable only to those couples in which at least one of the parents is a carrier of the ΔF508 mutation, or when both parents carry different CFTR mutations, other than ΔF508, because Elucigene CF29 can differentiate between the condition of heterozygote (carrier of a mutation) and homozygote (diseased) only in the case of ΔF508 mutation. Therefore, it is impossible to differentiate between heterozygotes and homozygotes for mutations other than ΔF508. In such a case, a complementary detection method is necessary, such as DNA sequencing.

At the same time, one cannot exclude the existence of other mutations besides the 29 mutations detected by Elucigene CF29, but these mutations have a much lower incidence than the 29 mutations that can be identified18, 19, 20,21,22

REFERENCES
REFERENCES (continued)