PRENATAL GENETIC DIAGNOSIS AND GENETIC COUNSELLING IN COUPLES AT HIGH RISK FOR MUCOVISCIDOSIS (CYSTIC FIBROSIS)

SUMMARY:
Mucoviscidosis is the most common genetic autosomal recessive disease in Caucasian populations, a potentially lethal disease and therefore prenatal genetic diagnosis is essential for couples with increased risk of having children with mucoviscidosis. Our goal was to detect CFTR mutations in fetal genomic DNA isolated from amniotic fluid collected by amniocentesis and to establish if the fetus is healthy, just a carrier for one CFTR mutation or both alleles are affected and the fetus has mucoviscidosis. Based on family history or echographic investigations, 11 couples were selected for prenatal genetic diagnosis. The couples had previously children with mucoviscidosis, registered at the National Center of Mucoviscidosis in Timisoara who had been genetically tested and had both mutations identified, or had deceased children who had been diagnosed with mucoviscidosis (with or 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 or by early amniocentesis in the 14th week of pregnancy. Contamination of fetal DNA isolated from amniotic fluid with maternal DNA was excluded by STR genotyping. Genomic DNA from the parents was isolated from venous blood collected on EDTA. The genetic analysis for CFTR mutations was performed using the Elucigen CF29 kit and electrophoresis of ARMS-PCR products in agarose gel.

The electrophoresis gels were visualized in UV light and after the interpretation of results, we identified 4 heterozygous genotypes \(\Delta F 508/N, G542X/N\), 6 normal genotypes and one compound heterozygote \(621+1 G>T/\Delta F 508\). Prenatal diagnosis can be performed by ARMS-PCR using Elucigen CF29 kit only in cases where the detected genotype has at least one allele with \(\Delta F 508\) 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 \(\Delta F 508\). The results obtained by prenatal diagnosis are essential for a complete and successful genetic counseling.

Key Words: mucoviscidosis, prenatal diagnosis, amniocentesis, CFTR mutations

DIGNOSTICUL GENETIC PRENATAL ȘI CONSILIEREA GENETICĂ A CUPLURILOR CU RISC CRESCUT DE MUCOVISCIDOZĂ

Rezumat:
Mucoviscidoză este cea mai comună boală genetică autosomarecesivă la populația caucasană, cu potențial letal și de aceea diagnosticul genetic prenatal este esențial la cuplurile cu risc crescut de a avea copii cu mucoviscidoză. Scopul nostru a fost de a detecta mutațiile CFTR în ADN-ul fetal genomic izolat din lichidul amniotic, colecțat prin amniocenteză și de a stabili dacă fetusul este sănătos, dacă este purtător a unei mutații CFTR sau ambele mutații sunt afectate și fetusul are mucoviscidoză. Baza pe istoria familiei sau pe ecografie am selectat 11 cupluri pentru diagnosticul genetic prenatal.

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**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), located on chromosome 7 in locus q31.2 . [1,2]

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, uriniferous tubules, genital ducts and sweat glands. [2]

Patients with mucoviscidosis suffer from a chloride ion conductance dysfunction, the CFTR 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 and 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. [3,4]

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). [5,6]

The correlation between the genotype of the patient and the expressed phenotype of the disease is variable and the clinical picture of mucoviscidosis patients may vary, depending on the existing CFTR mutations. The disease may have severe forms which can rapidly evolve to patient mortality, or milder forms with or without pulmonary and pancreatic involvement. On an average, patients that receive proper treatment since birth, survive up to the age of 20 – 30, therefore it is essential to establish a proper early diagnosis. [7,8]

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. [9,10,11]

Prenatal genetic diagnosis involves sampling of the amniotic fluid by amniocentesis. Amniocentesis is an invasive procedure which can be carried out between the 15 and 16th week of pregnancy or prior to the 15th week of pregnancy - early amniocentesis. The risk of fetal injury pregnancy loss is low (< 1.5%). [12]

**MATERIAL AND METHODS**

Eleven couples where selected for performing prenatal diagnosis. Eight couples 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 had deceased children clinically diagnosed with mucoviscidosis, with or without a molecular diagnosis. Three couples had fetal
hyperechogenic bowel diagnosed at routine ultrasonography but with no family history of mucoviscidosis. Genetic testing was carried out for each parent, regardless of their family history or previous genetic tests.\textsuperscript{[13]}

Molecular diagnostic was performed on genomic DNA isolated from venous blood samples collected on EDTA from both parents and on amniotic fluid samples collected by transabdominal amniocentesis in the 15th – 16th week of pregnancy. In one case amniotic fluid was collected by early amniocentesis in the 14th week of pregnancy (early amniocentesis). We collected 15 – 16 ml of amniotic fluid (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. By early amniocentesis only 600 il of amniotic fluid was collected. The amniotic fluid was also visually inspected after sampling to detect any possible traces of blood. 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 (2,000 rpm), the resulting supernatant (approximately 9 ml) was removed, the remaining sedimented fetal cells being resuspended in about 1 ml remaining fluid. These procedures aimed at concentrating fetal cells in the amniotic fluid and increasing the DNA quantity obtained following extraction. In the case of amniotic fluid collected by early amniocentesis we used the whole quantity without centrifugation of the sample. The blood samples taken from the parents were either immediately processed or preserved at -200\textdegree C for later analysis.\textsuperscript{[13]}

The Elucigene CF29 kit (Tepnel Diagnostics, UK) was used for detection of CFTR mutations. The kit can identify 29 mutations considered to be the most common among Caucasian populations: \(\Delta F\) 508, G542X, N1303K, 621+1G>T, 1717-1G>A, W1282X, 3849+10kbC>T, R553X, G551D, R117H, R1162X, R334W, A455E, 621+1G>T, 1717-1G>A, W1282X, 3849+10kbC>T, R553X, G551D, R117H, R1162X, R334W, A455E, 621+1G>T, 1717-1G>A, W1282X, 3849+10kbC>T, R553X, G551D, R117H, R1162X, R334W, A455E, 621+1G>T, 1717-1G>A, W1282X, 3849+10kbC>T, R553X, G551D, R117H, R1162X, R334W, A455E, 621+1G>T, 1717-1G>A, W1282X, 3849+10kbC>T, R553X, G551D, R117H, R1162X, R334W, A455E, 621+1G>T, 1717-1G>A, W1282X, 3849+10kbC>T, R553X, G551D, R117H, R1162X, R334W, A455E, 621+1G>T, 1717-1G>A, W1282X, 3849+10kbC>T, R553X, G551D, R117H, R1162X, R334W, A455E, 621+1G>T, 1717-1G>A, W1282X, 3849+10kbC>T, R553X, G551D, R117H, R1162X, R334W, A455E, 621+1G>T, 1717-1G>A, W1282X, 3849+10kbC>T, R553X, G551D, R117H, R1162X, R334W, A455E. For the AF508 mutation, in which case (patients have mucoviscidosis and both alleles are affected). 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 Elucigene CF29 kit use 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 \(\Delta F\) 508 mutation, in which case both the mutating sequence and the normal one can be amplified.\textsuperscript{[14,15]}

Genomic DNA was isolated from the lymphocytes found in the venous blood collected on EDTA and from amniocytes found in the amniotic fluid with a commercial kit - Qiagen QIAmp DNA Blood Mini. For the extraction of genomic DNA from the amniotic fluid, the extraction protocol (Qiagen) used for blood samples was slightly modified in order to enhance the concentration of isolated DNA. Thus, we used a greater volume of amniotic fluid (600 - 1200 \(\mu\text{l}\)), the final elution time was extended to up to 5 minutes and the volume of the buffer solution was reduced to 150 \(\mu\text{l}\) or even 100 \(\mu\text{l}\). DNA concentration was measured with NanoDrop 1000 \(\mu\text{l}\). For the blood samples the results were situated in the 20 – 60 ng/\(\mu\text{l}\) interval. For the amniotic fluid samples the results were situated in the 1 – 2 ng/\(\mu\text{l}\) interval. For the sample collected by early amniocentesis the DNA concentration was 1 ng/\(\mu\text{l}\). The recommended DNA concentration for an optimal PCR amplification is situated in the 1 – 10 ng/\(\mu\text{l}\) interval. We diluted the samples of genomic DNA extracted from blood in order to obtain an optimal concentration but the samples of fetal DNA isolated from amniotic fluid were used without dilution.

Isolated genomic DNA was amplified following the amplification program from Elucigene CF29 work protocol. For one sample, a PCR master mix which contained 1.5 \(\mu\text{l}\) DNA polymerase (Ampli Taq Gold), 8.5 \(\mu\text{l}\) sterile deionized water, 2.5 \(\mu\text{l}\) buffer solution for \(\mu\text{l}\)igation and 12.5 \(\mu\text{l}\) staining solution was constituted. The resulting mix was divided into four equal parts of 5.5 \(\mu\text{l}\) in four sterile 0.5 ml Eppendorf tubes, each tube containing 16.5 \(\mu\text{l}\) primer mixt (TA, TB, TC, TD). Subsequently, 20 were taken from each tube which were then introduced into a thin 0.2 ml PCR tube followed by 5 \(\mu\text{l}\) of the extracted genomic DNA. A DNA negative control was included in each set of PCR samples.\textsuperscript{[14]}

PCR amplification was conducted using the following amplification program: DNA AmpliTaq Gold polymerase
activation at 94°C for 20 minutes, followed by 35 cycles consisting of: denaturation at 94°C for 30 seconds, primer attachment stage at 580°C for 2 minutes and extension at 720°C for 1 minute. At the end of the amplification program, the extension stage of the last cycle, at 720°C, was programmed to last 20 minutes. [16]

The migration of PCR products obtained by amplification was carried out on agarose gel 3% (NuSieve 3:1; Cambrex BioScience) with ethidium bromide (20 μl ethidium bromide to 20 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) from which a dilution was made: 80 μl sterile deionized water, 10 μl staining solution and 10 μl Ladder 50 pb.[16]

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 to 1.5 hours).

The gels were visualized with an UV transluminator at 260 nm and photographed with a Canon Powershot A700 digital camera with filters adapted to the corresponding wavelength of ethidium bromide light emission [14,16]

The fluorescent signals for the sample collected by early amniocentesis were weak, but by increasing the exposure time when the gel was photographed we managed to obtain proper results which allowed us to establish the molecular diagnosis.

RESULTS AND DISCUSSION

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

In one 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 cu G542X mutation, thus the fetus being the carrier of this mutation (heterozygote). For the locus of the ΔF 508 mutation, the normal sequence was found, the mutant sequence characteristic for the ΔF 508 mutation being absent. The other electrophoresis bands represented the normal

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<td>Apo B 529bp</td>
<td>A555E 500bp</td>
<td>650</td>
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<td>621+1G&gt;T A383bp</td>
<td>2193AA&gt;G 425bp</td>
<td>600</td>
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<td>3126+1G&gt;A 443bp</td>
<td>550</td>
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<tr>
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<td>G551D 290bp</td>
<td>1898+1G&gt;A 280bp</td>
<td>500</td>
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<tr>
<td>G542X 279bp</td>
<td>R507 222bp</td>
<td>G551D 290bp</td>
<td>450</td>
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<td>R117H 243bp</td>
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<td>1148T 161bp</td>
<td>216dAIE 196bp</td>
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**Fig. 1.** Interpretation of the results obtained by electrophoresis of PCR products
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/ΔF 508 genotype (compound heterozygote). Both the ΔF 508 mutation and the G542X mutation are severe mutations which induce the occurrence of complete forms of the disease. Genetic testing of the parents showed that the mother was a carrier for ΔF 508 (genotype ΔF 508/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.

Three couples were carriers for 508 mutation (severe mutation) and had in their family history children with mucoviscidosis or deceased children of mucoviscidosis. In all these cases we established by prenatal diagnosis that the fetuses were only carriers for ΔF 508 mutation (genotype ΔF 508/N, figure 3) and the genetic counseling recommended the continuation of pregnancy. In one of these cases, due to the anatomical particularities of the mother it was not possible to collect an uncontaminated (with blood) sample of amniotic fluid. Therefore we isolated the fetal amniocytes by culture and the fetal genomic DNA was isolated from cultured amniocytes.

In one investigated couple, family history showed 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 father was a carrier of the ΔF 508 mutation, and the mother was a carrier of the 621 +1 G>T mutation. Prenatal diagnosis showed that the fetus was a compound heterozygote for the two mutations (genotype ΔF 508/621 +1 G>T), a condition that confirms the diagnosis of mucoviscidosis, both alleles being affected (figure 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. [19,20,21]

In only one case we used amniotic fluid collected by early amniocentesis. The volume of collected amniotic fluid was small (0.6 ml) and the interpretation of electrophoregram was difficult due to the reduced quantity of isolated DNA.

One important study which systematically looked at early amniocentesis was done in Canada (Canadian Early and Mid-Trimester Amniocentesis Trial (CEMAT) Group) published in 1998. In this trial, 4,374 women were randomized to either early amniocentesis (between 11 and 12 6/7 weeks) or midtrimester amniocentesis (between 15 and 16 6/7 weeks). This and subsequent reports from the trial demonstrated that compared to midtrimester amniocentesis, early amniocentesis was associated with a 4-fold risk of a technically difficult (twice the risk of requiring multiple needle insertions) or unsuccessful procedure (1.6% vs. 0.4%), a 10-fold risk of chromosome culture failure (2.4% vs. 0.25%), a higher rate of fluid leakage following the procedure (3.5% vs. 1.7%), a greater risk for pregnancy losses (7.6% vs. 5.9%), and a significantly higher risk (1.3% vs. 0.1%) of having a baby with talipes equinovarus (club foot). [22]

CONCLUSIONS

For a fast and accurate prenatal diagnosis in mucoviscidosis it is required to have 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.

Prenatal diagnosis can be performed on samples of amniotic fluid collected by normal amniocentesis in the 16th week of pregnancy or by early amniocentesis prior to the 15th week of pregnancy, however, due to the greater risks for pregnancy and fetus of this early procedure and because the volume of collected amniotic fluid is reduced (0.5 – 1 ml) it is recommended to perform normal amniocentesis. A small volume of amniotic fluid can offer only a small quantity of amniocytes for DNA isolation and the detection with Elucigene CF29 starting with a reduced concentration of isolated DNA can be difficult and inconclusive.

Mutation detection by ARMS-PCR with Elucigene CF29 is applicable only to those couples in which at least one of the parents is a carrier of the $\Delta F$ 508 mutation, or when both parents carry different CFTR mutations, other than $\Delta F$ 508, because the kit can differentiate between the condition of heterozygote (carrier of a mutation) and homozygote (diseased) only in the case of $\Delta F$ 508 mutation. Therefore, it is impossible to differentiate between heterozygotes and homozygotes for mutations other than $\Delta F$ 508. In such a case, a complementary detection method is necessary, such as DNA sequencing.

At the same time, we 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 identified.

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