PRENATAL GENETIC DIAGNOSIS IN MUCOVISCIDOSIS (CYSTIC FIBROSIS) BY CLASSIC AND EARLY AMNIOCENTESIS

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Abstract

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 classic amniocentesis and early amniocentesis in order to establish if the fetus is healthy, just a carrier for one CFTR mutation or both alleles are affected and the fetus has mucoviscidosis.

Key words: mucoviscidosis, CFTR mutations, amniocentesis.

Introduction

Mucoviscidosis or cystic fibrosis is caused by mutations of the CFTR gene (cystic fibrosis transductance conductance regulator), located on chromosome 7 in locus q31.2. Over 1600 mutations and sequence variations (polymorphisms) have been described until now, however only one mutation was found in over 70 % of the investigated chromosomes - ΔF508. 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 22 persons[1,2].

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 (most commonly between 12–14 gestational weeks). Early amniocentesis results are available 4–6 weeks before standard amniocentesis and 1–3 weeks after chorionic villus sampling (CVS), presenting an attractive method for prenatal diagnosis in the early second trimester, despite a somewhat higher rate of immediate post procedure complications[11,12,13].

Amniocentesis should be preceded by genetic counseling, in which the family pedigree and genetic risk are evaluated and the advantages and risks of the procedure are explained.

Material and methods

Ten couples where selected for performing prenatal diagnosis. Eight couples were carriers for CFTR mutations. The couples 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. Two couples had fetal hyperechogenic bowel diagnosed at routine ultrasonography. Genetic testing was carried out for each parent, regardless of their family history or previous genetic tests.

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 classic amniocentesis (16th week of pregnancy) in 9 cases and by early amniocentesis (13th week of pregnancy) in one case.

By classic 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.

The amniotic fluid was also visually inspected after sampling to detect any possible traces of blood. Brown or green tinged amniotic fluid is aspirated in 1–6% of midtrimester amniocenteses and may be associated with an increased risk (5–9%) of perinatal mortality and pregnancy loss. Analysis of discolored fluid samples indicates that in most cases the discoloring pigment is hemoglobin. Vaginal bleeding prior to amniocentesis seems to predispose for presence of discolored amniotic fluid[13].

By early amniocentesis only 600 µl of amniotic fluid was collected. It has been suggested that removal of amniotic fluid at amniocentesis, especially when performed early in gestation, may affect fetal lung development. Lung function tests performed after birth to babies subjected to amniocentesis apparently demonstrated lower dynamic compliance and higher resistance compared to controls. Other studies could not document an effect on neonatal lung

function tests but noted a significantly higher incidence of respiratory distress and admissions to special care units for neonates subjected to chorionic villus sampling (CVS) in the first trimester. Apparently, both amniocentesis and CVS performed in the first trimester may impair antenatal lung growth. Therefore only a reduced volume of amniotic fluid was collected[13].

DNA was isolated immediately after sampling in order to obtain the best results. Due to 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 -20^0C for later analysis[14,15].

For detection of CFTR mutations we used the Elucigene CF29 kit (Tepnel Diagnostics, UK). The kit can identify 29 mutations considered to be the most common among Caucasian populations. 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 (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 ARMS-PCR (amplification refractory mutation system)[15,16].

For genomic DNA isolation from blood collected on EDTA and from amniocytes found in the amniotic fluid we used 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 µl), the final elution time was extended to up to 5 minutes and the volume of the buffer solution was reduced to 150 µl or even 100 µl. DNA concentration was measured with NanoDrop 1000. For the amniotic fluid samples the results were situated in the 1 – 2 ng/µl interval and for the sample collected by early amniocentesis the DNA concentration was 0,5 - 1 ng/µl. The recommended DNA concentration for an optimal PCR amplification is situated in the 1 – 10 ng/µl interval. The samples of fetal DNA isolated from amniotic fluid were used without dilution[14,15,16,17].

Isolated genomic DNA was amplified following the amplification program from Elucigene CF29 work protocol: AmpliTaq Gold polymerase activation at 94^0C for 20 minutes, followed by 35 cycles consisting of denaturation at 94^0C for 30 seconds, primer attachment stage at 58^0C for 2 minutes and extension at 72^0C for 1 minute. At the end of the amplification program, the extension stage of the last cycle, at 72^0C, was programmed to last 20 minutes. For one sample we used a PCR master mix which contained 1.5 µl DNA polymerase (Ampli Taq Gold), 8.5 µl sterile deionized water, 2.5 µl buffer solution for dilution and 12.5 µl staining solution. The resulting mix 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 mixt (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[14,15]. Electrophoresis of PCR products 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. We used the 50 Base-Pair Ladder (Amersham-Pharmacia Biotech) as marker of the size of the fragments obtained by PCR reaction (dilution: 80 µl sterile deionized water, 10 µl staining solution and 10 µl Ladder 50 bp). In each well of agarose gel 20 µl of the PCR products were introduced, and adjacent to these we loaded 20 µl 50 Base-Pair Ladder dilution[14,15]. The migration 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)[16,17]. The gels were visualized with an UV transluminator at 260 nm and photographed with a Canon Powershot A710 digital camera with filters adapted to the corresponding wavelength of ethidium bromide light emmision. 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[15,16,17,18].

Results and discussion

The photos of the gels were analyzed and the results were interpreted according to the diagram shown in Figure 1.

Genetic testing of the parents in one investigated couple showed that the mother was a carrier for ΔF508 mutation (genotype ΔF508/N), and the father was a carrier of the G542X mutation (genotype G542X/N). The couple had already had a child with mucoviscidosis[19,20], complete form, with pulmonary and pancreatic involvement, who was genetically tested and presented the G542X/ΔF508 genotype (compound heterozygote). Both the ΔF508 mutation and the G542X mutation are severe mutations which induce the occurrence of complete forms of the disease.

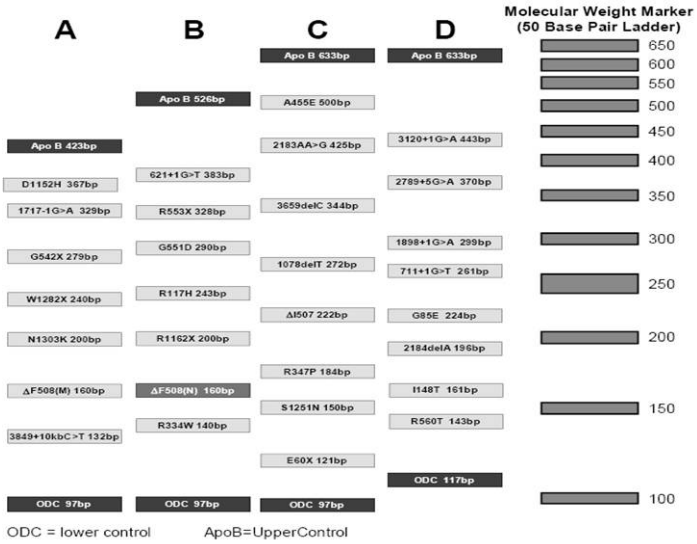

Figure 1. Interpretation of the results obtained by electrophoresis of PCR products.

The electrophoregram for this case showed the presence of a 279 pb fragment which, according to the interpretation diagram, corresponds with the G542X mutation. For the locus of the ΔF508 mutation, the normal sequence was found (160 bp), the mutant sequence characteristic for the ΔF508 mutation being absent (Figure 2). The fetus was only a carrier of G542X mutation (heterozygote). As for the disease to become manifest requires the existence of at least two mutant alleles, the fetus was going to be clinically healthy. The couple was informed the fetus health condition, and the recommendation made during genetic counseling was to continue the pregnancy[15].

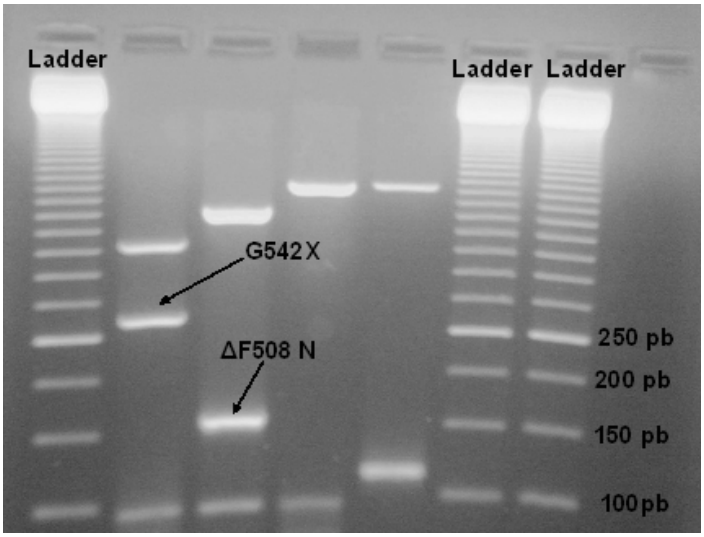

Figure 2. Electrophoresis of PCR products – G542X heterozygote, G542X/N genotype.

Three couples were carriers for ΔF508 mutation (severe mutation) and had in their family history children with mucoviscidosis or deceased children of mucoviscidosis[21,22]. In all these cases we established by prenatal diagnosis that the fetuses were only carriers for ΔF508 mutation (genotype ΔF508/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 deceased child, who had died in its first month, clinically diagnosed with mucoviscidosis but without molecular diagnosis. The parents were genetically tested and the results showed that the father was a carrier of the ΔF508 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 ΔF508/621+1G>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[15].

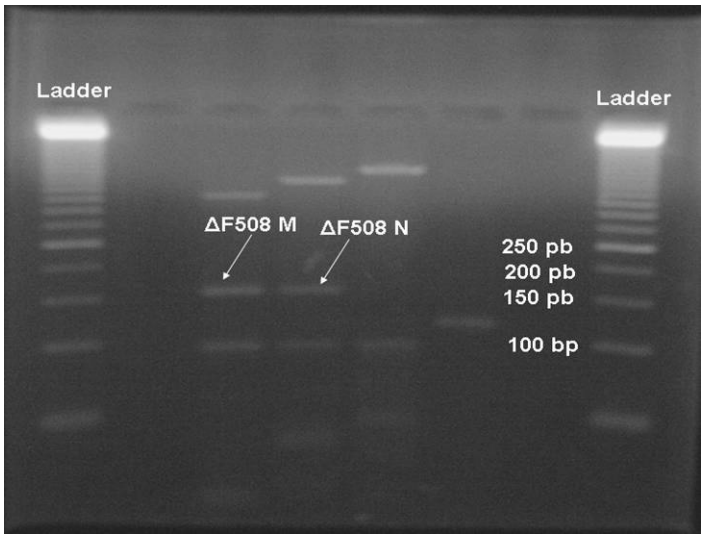

Figure 3. Electrophoresis of PCR products – heterozygous genotype, ΔF508/N.

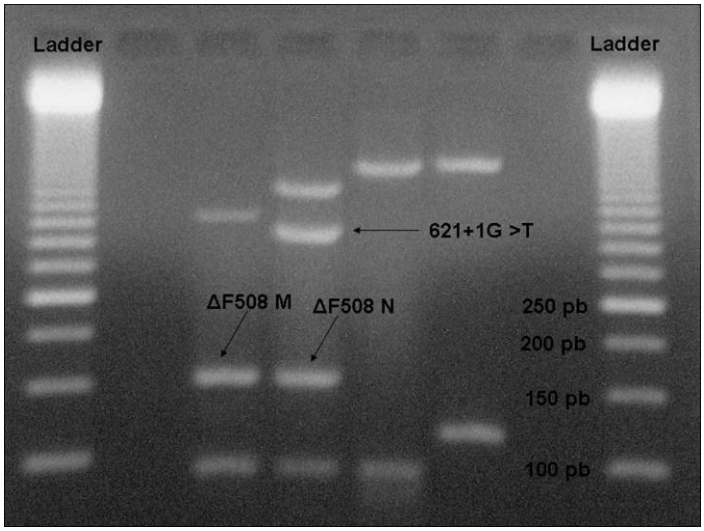

Figure 4. Electrophoresis of PCR products – compound heterozygote, genotype ΔF508/621+1 G>T.

A normal genotype was found in the prenatal genetic diagnosis for 5 couples. The ΔF508 mutation or other mutations were absent. In figure 2 and figure 3 we can observe the electrophoresis band characteristic for the normal sequence for the ΔF508 locus (ΔF508 N, 160 pb). The parents were previously tested. In one couple the mother was 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, who was not 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.

In other couple the mother was a carrier for N1303K mutation and the couple had a child with mucoviscidosis who was genetically tested but only one mutation (N1303K) was found. Since it was possible that the father was a carrier for a CFTR mutation which could not be detected by Elucigene CF 29, the only variant which was acceptable for the fetus was a healthy genotype, situation which was found in this case and the recommendation was to continue the pregnancy.

Two couples had echographic findings (hyperechogenic bowel) which can be a hallmark for mucoviscidosis and recommended the prenatal diagnosis. However in these cases the results were negative for mucoviscidosis and a normal fetal genotype was found.

In only one case we used amniotic fluid collected by early amniocentesis. Both parents were carriers of ΔF508 mutation and had a deceased child with mucoviscidosis. The volume of collected amniotic fluid was small (0,6 ml) due to the risks of the procedure to the fetus and the interpretation of electrophoregram was difficult due to the reduced quantity of isolated DNA. Weak fluorescent signals were photographed on the gel and we had to increase the exposure time in order to have suitable results (Figure 5).

Improved ultrasound technology, increasing experience with ultrasound-guided needle manipulation and patient preference for more private, earlier genetic diagnosis have motivated a shift from second trimester amniocentesis toward earlier procedures - CVS and “early” amniocentesis.

Early amniocentesis refers to procedures performed before 15 weeks' gestation (most commonly between 12–14 gestational weeks). The approach technique is somewhat different from that used at midtrimester, for 2 reasons. First, ultrasound guidance is essential as the size of the fluid pocket is much smaller and requires greater experience to access safely. Second, if one pushes the needle slowly into the pocket, there is a much higher likelihood of tenting the fetal membranes, which did not adhere yet to the uterine wall. A 22-gauge needle was used, which is inserted to the myometrium. After fetal position is verified, the needle is advanced in a single, swift thrust into the pocket of fluid. Sometimes, rotating the needle may also help to overcome tenting of the membranes. One ml of amniotic fluid per every week of gestation is aspirated into a syringe, transferred into sterile tubes and sent to the laboratory for processing[13].

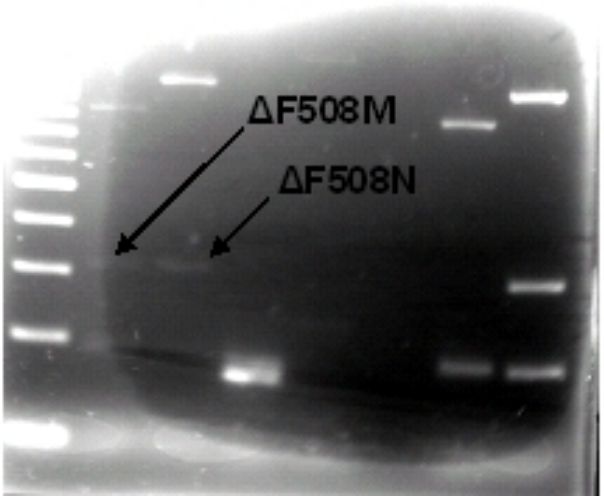

Figure 5. Electrophoregram of PCR products obtained by amplification of isolated DNA from amniotic fluid collected by early amniocentesis. Increased exposure time of photo.

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[23]. 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]. Early amniocentesis (EA) was considered as an early prenatal diagnosis technique option due to the concerns related to CVS and the wide spread use of amniocentesis at 15–16 weeks gave false reassurance that amniocentesis could be used safely at an earlier gestational age. The ultrasound-guided amniocentesis technique was moved down in gestational age with procedures being undertaken in the 11–14 gestation weeks. Early observational studies were not able to identify the risks of the procedure and it was only after 3 randomized trials were completed (Sundberg, 1997; CEMAT, 1998 and EATA, 2004)[23,24,25] that the true risks of the procedure were identified. These early amniocentesis risks included higher total pregnancy loss, a significant increase incidence of musculoskeletal foot deformity, a significant increased culture failure rate, and an increased post amniocentesis rate of leakage compared with the gold standard mid-trimester amniocentesis. The early amniocentesis window considered in these randomized trials was 11 weeks and 0 days to 13 weeks and 6 days. The specific risk of EA in a gestational window of 14 weeks 0 days to 14 weeks 6 days has not been clearly defined[13].

Conclusions

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 14th 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 classic amniocentesis.

Early amniocentesis is attractive because of a shorter learning curve, the availability, the low rate of maternal-cell contamination and the early gestational timing.

Also, for an 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 maternal cells, and a good technique for DNA isolation.

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 ΔF508 mutation, or when both parents carry different CFTR mutations, other than ΔF508, because the kit can differentiate between the condition of heterozygote (carrier of a mutation) and homozygote (diseased) only in the case of ΔF508 mutation.

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