The use of DHPLC (Denaturing High Performance Liquid Chromatography) in II level screening of the CFTR gene in Prenatal Diagnosis

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Summary
The aim of the study is to evaluate the role of Denaturing High Performance Liquid Chromatography (DHPLC) in the second level screening of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene. Methods. A 9-month prospective study, between June 2008 and March 2009 at Artemisia Fetal Medical Centre, included 3829 samples of amniotic fluid collected from women undergoing mid-trimester amniocentesis.
The genetic diagnosis of CF was based on research of the main mutations of the CFTR gene on fetal DNA extracted from the amniocytes, (first level screening) using different commercial diagnostic systems. A second level screening using DHPLC, on the amniotic fluid and on a blood sample from the couple, was offered in case of fetuses heterozygous at first level screening. Results. Of 3829 fetuses, 134 were found to be positive, 129 heterozygous and 5 affected. Of the 129 couples, following appropriate genetic counselling, 53 requested a second level screening. Through the use of DHPLC, 44 couples were found to be negative, and in nine couples, nine rare mutations were identified. Conclusions The first level screening can be useful to evidence up to 75% of the CF mutations. The second level screening can identify a further 10% of mutant alleles. DHPLC was found to be a reliable and specific method for the rapid identification of the rare CFTR mutations which were not revealed in initial first level screening.

Key words: CFTR, gene, Cystic fibrosis, Chromatography

Background

Cystic Fibrosis (CF) is a multi-systemic illness characterized by mutations of the CFTR gene, which regulates chlorine, sodium and bicarbonate secretion in the epithelial tissues. The principal symptoms of the illness regard the lungs, insufficiency of the exocrine pancreas, male sterility and excessive sweating. Today, there are more than 1500 existing mutations of the CFTR gene and numerous polymorphisms (> 200), which are important from a functional point of view. (1,2). The early identification of the illness is important in order to be able to take appropriate therapeutic action, offer genetic consultancy and guarantee access to specialized medical services. However, in some ethnic groups, diagnosis through the screening of direct mutations has been shown to be inconclusive due to a high allelic heterogeneity and variable frequency of pathogenetic mutations.

Introduction

Cystic Fibrosis (OMIM # 219700) is one of the most common genetic illnesses of the Caucasian population with a frequency of 1/2500-3500.(3,4). The people affected by the illness are homozygous for the same mutation or heterozygous for differing mutations. The gene is contained in a region of DNA which is 230 Kb in length, and which contains 27 exons and codifies for a protein of 1480 amino-acids.(5,6). The protein, which is highly conserved in various species, belongs to a large family of glycoproteins which possess intracellular sites linking with ATP and which carry out essential functions in active ion transport. For this reason, it has been defined "cystic fibrosis transmembrane conductance regulator" (CFTR), (7,8). The CFTR gene is composed of two transmembrane domains TM1 and TM2, two intracellular sites linking with ATP (NBF: Nucleotide Binding Fold) and an intracellular regulatory region (R), which separates the two protein domains.
To date, more than 1500 mutations and more than 200 polymorphisms have been identified, in both codifying and non-codifying regions. The mutations can be found along the entire structure of the CFTR gene. However, there are "hot sites" corresponding to essential protein

domains for the correct functioning of the CFTR canal (9,10).
The most common mutation regards the elimination of three nucleotides (1652delCTT) which results in the loss of a residue of phenylalanine in the 508 position (F508del) of the genic product. (11) The F508del mutation is present in approximately two thirds of the CF chromosomes, with Italian percentages which are very variable compared to frequencies in the European population. There is a group of particularly representative mutations which have been described by the CF Managing Committee in 2001, which occur at a European frequency of 0.1 %. The remaining mutations are defined as "rare", and found in few individuals. (12) The phenotype of Cystic Fibrosis is very variable, ranging from a "mild" symptomatology, such as idiopathic pancreatitis, chronic sinusitis, nasal polyposis, asthma, bronchiectases and congenital bilateral absence of the deferential vessels (CBADV), to a more serious and aggressive phenotype, associated with respiratory and digestive symptoms. (13). The principal clinical symptomatology of the serious forms regards the respiratory apparatus which starts during the first few months of life, and the obstruction of the mucosa of the respiratory passages, which develops into bronchiectasis. The respiratory epithelia secrete bacterial substances whose activity depends on salt concentration (sodium and chlorine), which is particularly high in patients. This inhibits any form of bactericidal activity and determines an environment which favours bacterial infection. Infections caused by Staphylococcus aureus, Pseudomonas aeruginosa and Candida albicans are very common. The obstructive chronic lung illness determines a progressive modification of the cardiac functioning and this can develop into chronic cor pulmonale. In both sexes, there can be alterations of the reproductive system. (14,15)
CF segregates as an autosomal recessive mutation, with risks of recurrence in 1 out of 4 children with heterozygous parents and a proportional increase in reproductive risk for the blood relations of the patients.
The clinical diagnosis of CF is based on phenotypic criteria, family history, and on a positive test for hypertrypsinemia (IRT) in the neonatal period. In most cases, the diagnosis of CS is confirmed by high levels of chlorine highlighted through a sweat test (> 60 nmol/l). This molecular diagnosis is based on research into the principal mutations of the CFTR gene. Various methods have been adopted for the identification of the sequence variations of the entire codifying region of the CFTR gene (such as, for example, the SSCP, or the DGGE method). In recent years, a number of groups have used the DHPLC technology to identify FC alleles. (15,16)

Materials and methods

During the pre-amniocentesis meeting which took place in our Centre of Prenatal Diagnosis, the pregnant women were free to choose whether to request screening or not also for the most frequent mutations of the CFTR gene, which was carried out on the same amniotic fluid taken for the study of the karyotype. From 1997 to 2007, the number of pregnant women that requested the 1 level screening for CF mutations increased constantly. Today, in 2009, the figure is around 75 %. Only 3 – 5 % of these requests are made due to family risk, while 2 – 3% are due to evidence of certain characteristics emerging from a foetal echography: meconium ileus and typical eco-refraction, dilation of the distal intestine and obstruction at the caecum. Most pregnant women request screening for personal reasons and for peace of mind.
With this rise in requests for screening in recent years, there has been an attempt to increase the detection rate of CFTR mutations, improving I level screening and using different commercial diagnostic systems such as the Reverse Dot Blot (RDB) of the INNOGENETICS (CFTR19 – CFTR17 – Italian Regional), or the OLA system of the ABBOTT, and, since June 2008, using DHPLC (Denaturing High Performance Liquid Chromatography), for II level screening. In fact, for all patients where the foetus was found to be heterozygous for the CFTR gene at I level screening, further screening was proposed at II level through DHPLC. (16,17).
The I level screening was carried out only on foetal DNA extracted from the amniocytes.
The screening at II level was carried out on the amniotic fluid and also from a hematic sample from the couple. Our study covered a monitoring period of nine months which started on 01 June 2008 and ended on 01 March 2009. In this period, 3829 samples of amniotic fluid were taken from different amniocenteses, 134 of which were found to be positive, 129 heterozygous healthy carriers and 5 foetuses were found to be affected. Through the use of commercial kits, it was possible to analyse a varying percentage of mutations, which has increased through the years, to between 70 % and 75 % of frequencies in the Italian population. Of the 129 couples for whom a heterozygous foetus was identified for a mutation of the CFTR gene, following appropriate genetic counselling, 53 requested screening at II level through DHPLC. Following this, the residual risk of the foetus being affected passed from approximately 1/400, foreseen with I level screening, to approximately 1/1800 after screening at II level. (17)

Amplification of the DNA

For research into the principal mutations, like for screening at I level, we used the Abbott system – OLA PCR (Oligonucleotide Ligation Assay). Amplification of the regions involved (intron/Exon), the sites of the 32 mutations being researched, took place through 31 amplification reactions using specific primers, according to Marechal et al. [17]. The subsequent controls through the use of DHPLC were preceded by a reaction of PCR, utilizing from 50 to 100 ng of DNA extracted from amniotic fluid. Amplification was performed in a reaction volume of 25 µl which contained 0.2 mM of primers, 200 µM of dNTPs, 1 X buffer (tris HCl pH 8.3, 50 mM, KCl, 2.5 mM MgCl2 10 mM) and 1.25 U AmpliTaq Gold™ DNA polimerase (Applied Biosystems, Foster City, CA), using a DNA amplifier (Applied Biosystems, Foster City, CA). The PCR was carried out with an initial denaturation of 94° C for 10 min followed by 35 cycles at 94° C for 40 sec, from 57° C or 61° C or 54° C for 40 sec and 72° C for 1 min, followed finally by 7 min at 72° C. (14,15) The amplification products were controlled through electrophoresis on agarose gel before analysis through DHPLC.

DHPLC analysis

DHPLC technology is currently considered one of the most reliable techniques for identifying mutations, insertions, deletions, and other molecular variants. It is a separation technique able to resolve the components of a mixture, in this case molecules of DNA, into a special chromatographic column, modulated by the combined effects of temperature, buffers, type of sequence etc. (18)
The analysis with DHPLC was carried out on the DNA WAVE system of the producer (Transgenomic™, Crewe, UK), equipped with a® DNASep column (Transgenomic™, Crewe, UK). Following amplification, 5 ul of each PCR product was denatured at 95° C for 5 minutes, and renatured slowly (1°C/min) up to a temperature of 60°C, and then loaded onto the chromatographic column. The DNA was eluted from the column thanks to a linear gradient of acetonitrile 0,1 mM, and a constant flow of 0,9 ml/min. (18, 19, 20) The gradient was created by mixing buffer A (0,1 M TEAA) and buffer B (0,1 M TEAA, acetonitrile 25 %). For each fragment, the initial and final concentrations of buffer B were calculated in order to obtain a retention time of between 3 and 5 minutes. The temperature required for the optimum separation of the heteroduplexes, in partial denaturation conditions, is calculated starting from the melting profile of the DNA sequence. Il WAVEMAKER 3.4.4. software was first used to calculate melting curves, and then to estimate the temperature of analysis. (18, 19, 20)

Analyses of the data and results

The screening of the principal mutations (I level) was performed using the OLA methodology (Oligonucleotide Ligation Assay) of the ABBOTT which, through the study of 32 mutations, made it possible to exclude approximately 72 % of those present in the Caucasian population. The mutations that were excluded with I level screening are reported in table I.
Table II contains the mutations found with I level screening for 53 heterozygotes.
The study with DHPLC (II level screening) was developed in order identify documented or unknown mutations, which added to the mutations already found, determine the CF phenotype. For the 53 couples that requested analyses, informative counselling was provided and at the same time a hematic sample was taken from both partners of every couple. Once the partner carrying the foetal mutation had been identified, the other member of the couple, being found negative at I level screening, had their codifying region of the CFTR gene analysed using DHPLC.
Through the use of DHPLC, all the exonic regions of the CFTR gene were analysed and through the technique 44 of the 53 couples were found to be negative, while for 9 couples, 9 rare mutations were identified which were not revealed in I level screening: R1066C, L1065P, L1077P (exon 17b), A1006E (exon 19), R75Q (exon 3), D537E (exon 11), W1134X (exon 18), R1145X (exon 18), C524X (exon 11). The unknown mutations, therefore, were identified using DHPLC technology. Figures 1, 2, and 3 show some of the anomalous chromatographic profiles. Differently from the normal control which shows one single peak of homoduplex DNA, all the samples which at exon level had a different chromatographic variant were sequenced automatically. The direct sequencing of the single exons was carried out using the automatic ABI PRISM 3100 sequencer (PE Applied Biosystems), utilizing the technology of the Big Dye Terminator. The DHPLC conditions were prepared according to the WAVEmaker software (Transgenomic)

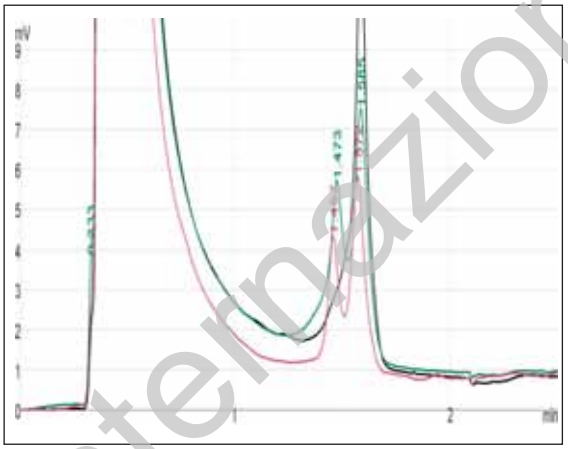

Fig. 1 - Chromatographic analysis of exon 10.

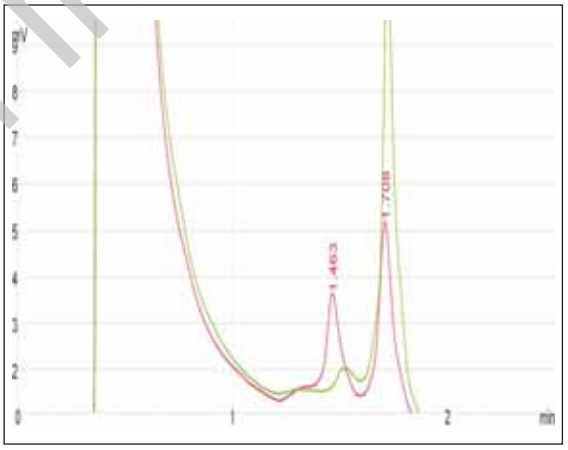

Fig. 2 - Chromatographic analysis of exon 4.

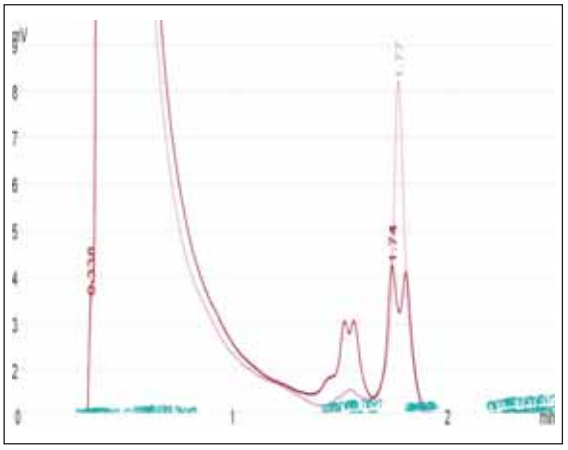

Fig. 3 - Chromatographic analysis of exon 21.

Table II **Mutations found through I level screening**

Mutations	*Positions on CFTR gene*
R1066C	**Exon 17 b**
L1065P	**Exon 17 b**
A1006E	**Exon 19**
R75Q	**Exon 3**
D537E	**Exon 11**
W1134X	**Exon 18**
W1145X	**Exon 18**
L1077P	**Exon 17b**
C524X	**Exon 11**
Total	**9**

Table I **Mutations found through I level screeningMutations analysed with I level screening through OLA**

CFTR Mutations	**Position on the CFTR gene**
DF508	**Exon 10**
3849+10KbC®T	**Intron 19**
R334W	**Exon 7**
W1282X	**Exon 10**
V520F	**Exon 10**
3905insT	**Exon 20**
N1303K	**Exon 21**
3876delA	**Exon 20**
1717-1G®A	**Exon 11**
3659delC	**Exon 19**
DI507	**Exon 10**
A455E	**Exon 9**
G85E	**Exon 3**
2789 +5G®A	**Exon 14 / Intron 14**
2183AA®G	**Exon 13**
1898+1G®A	**Exon 12 / Intron 12**
R347P	**Exon 7**
R347H	**Exon 7**
R560T	**Exon 11**
1078delT	**Exon 7**
R553X	**Exon 11**
711+1G®T	**Exon 5 / Intron 5**
G551D	**Exon 11**
R1162X	**Exon 19**
S549R	**Exon 11**
R117H	**Exon 4**
S549N	**Exon 11**
621+1G®T	**Exon 4**
G542X	**Exon 11**
394delTT	**Exon 3**
3120+1G®ðA	**Exon 16/ Intron 16**
2184delA	**Exon 13**

Table III **Mutations found with II level screening through DHPLC**

Mutations	of mutated alleles
DF508	**29**
W1282X	**3**
N1303K	**8**
1717-1G®A	**2**
3659delC	**1**
G85E	**1**
2789 +5G®A	**2**
R553X	**2**
R1162X	**1**
R117H	**1**
G542X	**3**
Total	**53**

and the DHPLC programme. (18,19,20) In the 53 CF mutated alleles, in total, through 1 level screening, 11 different mutations were evidenced which represent approximately 73 % of CF chromosomes. The complete spectrum of mutations revealed in the sample is shown in table III. The 9 rare mutations found with II level screening increased the percentage of identified CF alleles by approximately 10%. The I level screening can be useful to evidence up to about 75 % of the CF mutations and can be carried out with various technological systems which are available on the market. The II level screening of the CFTR gene with DHPLC presented in the present study made it possible to identify a further 10 % of mutant alleles, in particular another 9 rare mutations. The screening of mutations in new-born children which clinically are suspected to have cystic fibrosis can help confirm such diagnoses and in some cases can make it possible to foresee the seriousness of the illness. In this way, it can be possible to organize early treatment and delay the onset of irreversible damage so guaranteeing the maximum possible quality of life.

Conclusions

In this study, using DHPLC, we analysed the entire codifying region of the CFTR gene in the genomic DNA obtained from a cohort of 53 pregnant CF patients with a foetus that was found to be heterozygous with I level screening. Of these 53 patients, 44 were found to be negative following DHPLC, while 9 patients were found positive for another 9 rare mutations, which, in addition to those mutations already identified with I level screening, determined the presence of a heterozygous foetus for the CFTR gene and therefore the illness. From a technical point of view, further confirmation of the anomalous chromatographic variant is required through direct sequencing. On the whole, DHPLC was found to be a reliable and specific method for the rapid identification of the rare CFTR mutations which were not revealed in initial I level screening and it would also be particularly suitable for identifying unknown mutations.

Regarding the evaluation of the results obtained during pregnancy, the principal aspect to take into consideration was to provide the couple with adequate counselling in order to better understand the genotype-phenotype correlation in the various associations of mutations. The genetic counselling was not provided only in order to explain the residual percentage risk and provide rational evaluations based on numbers and statistics. In fact, during the meetings, the couples were also given assistance in order to help them come to terms with a pregnancy in which the suspicion of cystic fibrosis unexpectedly appears.

The fundamental aspect of the counselling was to reassure couples when deciding whether or not to terminate pregnancy and whether even a low risk of CF was acceptable when opting to proceed with the pregnancy. In other words, the couple were assisted, in a moment of the pregnancy and based on the results of the investigations, in deciding on whether it was acceptable to continue pregnancy, despite a degree of worry and apprehension. This possibility was also discussed during counselling. However, causing this worry is justifiable considering the fact that discovering the seriousness of the illness before birth can facilitate the organization of early treatment and delay the onset of irreversible damage so assuring the new-born children a better quality of life. The level of screening, therefore, appears to be also of great importance in prenatal diagnoses. The routine methods, INNO-LiPA CFTR, OLA etc., are based on a selection of known mutations, also established on a geographical basis and their frequency is variable between various ethnic groups. Therefore, it is difficult to develop an appropriate policy for all the populations that cover all the known mutations. For this reason, following I level screening using standard kits, further investigation with DHPLC can constitute a method which overcomes the problems related to geographical distribution. We have found that the DHPLC technique is also a reliable and specific method which can be adopted in prenatal diagnosis to discover quickly mutations of the CFTR gene which cover more than 90 % of the CF alleles in our population but which do not exceed 95 % because larger genomic rearrangements or different mutations cannot be identified or visualized with this method. Therefore, considering the above, we suggest caution in using this diagnostic system and in cases where it is considered necessary (accompanied by genetic counselling) also consider scanning of the CFTR gene through III level screening.

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