Original Article

Prenatal diagnosis for CF using High Resolution Melting Analysis and simultaneous haplotype analysis through QF-PCR☆,☆☆,★

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Abstract

Background: High Resolution Melting (HRM) Analysis is a validated, robust, low-cost, high throughput CF screening method. Here, we report the development and retrospective evaluation of the diagnostic value of a novel multiplex HRM, genotyping and haplotyping method for CF prenatal diagnosis (generic HRM/haplotyping).

Methods: 80 study samples from 20 carrier couples referred for PND (whole blood in EDTA and CVS or amniotic fluid) were genotyped retrospectively using the suggested protocol.

Results: All DNA samples (variable sources, extraction methods and unknown concentrations) were successfully amplified by the 1st and 2nd round PCR. The Se, Sp, NPV and PPV for the generic HRM/haplotyping method are calculated at 100%.

Conclusions: This generic protocol for PND using HRM, facilitates the simultaneous analysis of DNA samples from various sources in a fast, robust and efficient way. It can be easily adapted and applied for any genetic condition.

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Keywords: Cystic fibrosis; High Resolution Melting; Prenatal diagnosis; Mutation detection; Haplotype analysis; QF-PCR

1. Introduction

Cystic fibrosis (CF) (MIM#219700) is the second most common autosomal recessive disorder in Greece after thalassemias. The carrier frequency is estimated to be approximately 3–4% of the general population with an incidence of about 1 in 2500–3500 live births [1]. Cystic fibrosis is caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene. CFTR is located on chromosome 7q31.2, encoding a glycosylated transmembrane protein of 1480 amino acids. The gene has been identified in 1989 [2], and since then more than 1800 disease causing variants have been described [3]. The frequency and distribution of disease causing variants show a clear ethnic and geographical distribution. The frequency of the most common mutation p.Phe508del (F508del, c.1521_1523delCTT) (CFTR reference sequence NM_000492.3 and NG_016465.1) varies significantly between ethnic groups, even between countries of close geographic proximity. In Greece the frequency is estimated at ≈53% of CF alleles, whereas in neighbouring Albania the frequency is ≈73%.

☆ Gene Symbol: CFTR.
☆☆ Approved name: cystic fibrosis transmembrane conductance regulator (ATP-binding cassette sub-family C, member 7).
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[1] (our unpublished results). In Europe the frequency of the commonest mutation may vary from as high as 87.5% in Denmark to 36.6% in Romania [1]. Regardless of the frequency, p.Phe508del is always screened for, when testing for CFTR mutations.

The typical presentation of CF includes progressive pulmonary disease, pancreatic insufficiency, meconium ileus, liver disease with advancing age and positive sweat chloride test [4]. Although quality of life and expectancy have greatly improved over the past decades for all CF patients, CF remains a severe and incurable disease, therefore prenatal diagnosis (PND) is offered to all couples when both members are known carriers of severe CF mutations.

The Greek population has one of the highest rates of CFTR mutation heterogeneity, making molecular diagnosis especially difficult [5]. Due to the degree of heterogeneity the highest detection rate that can be achieved by commercially available kits, reaches less than 80% [6]. In order to achieve a higher detection rate and since the Department of Medical Genetics (DMG) serves as a reference laboratory, we have been performing exon scanning using DGGE (Denaturing Gradient Gel Electrophoresis) followed by bi-directional sequencing of all positive findings [2]. However DGGE is a very laborious and time consuming methodology which makes it incompatible with the increasing number of samples being analysed. For these reasons, in the past three years, we have changed our scanning methodology to High Resolution Melting Analysis (HRM) using LightScanner® (Idaho Technologies, Utah, USA).

HRM is based on a fundamental property of DNA which is melting. The method consists of PCR amplification of the fragment in question, in the presence of a dsDNA intercalation fluorescent dye (LCGreen® Plus, Idaho Technologies, Utah, USA). Following PCR the samples are melted. Heating of the double helix, causes the separation of the two strands creating a unique melting curve pattern according to the composition of the fragment analysed. Any change in the composition of the fragment will create a different melting profile allowing the user to distinguish between the variants [7]. HRM is an inexpensive, fast and robust method for detecting sequence changes in genomic DNA.

In our laboratory HRM has been validated and is currently being used as a routine method for screening the whole coding sequence and flanking intronic regions of the CFTR gene. Since the application of HRM for CFTR screening, we have analysed more than 4000 samples with exceptional sensitivity and specificity rates. The primers used are as published in the paper from Montgomery et al. [8]. All positive findings are confirmed by subsequent bi-directional sequencing. Samples that do not clearly fall in the wild type genotype (borderline findings) are re-analysed in a second HRM experiment and if necessary the genotype is confirmed by sequencing.

A successful HRM experiment depends on primer (gene scanning) and probe (genotyping) designs as well as DNA quantity and quality. One major drawback of the method is that it requires standardised DNA extraction and purification so that all samples are finally dissolved in the same buffers to minimize ionic differences that affect melting curves [9]. That signifies that the use of samples from different sources (peripheral blood, amniotic fluid and chorionic villus samples), different extraction protocols (salting out, manual extraction kits and robotic extraction systems) or poor quality DNA (samples with impurities) in the same experiment is prohibitive.

These limitations pose a serious problem in a diagnostics laboratory that also serves as a reference laboratory for molecular analysis of the CFTR gene. Our laboratory often receives not only blood samples, but also already extracted DNA samples from other laboratories in order to further analyse them. These samples cannot be analysed in the same experiments and it is very difficult to create batches of samples extracted in the same way since each laboratory uses different DNA extraction protocols. When providing diagnostic services each positive finding has to be confirmed by two methods. Since we have switched from DGGE analysis it is not feasible to use the old technology to analyse these samples. Therefore the need arose for the establishment of a second method, besides sequencing, to confirm the results.

These problems also apply to prenatal diagnosis where ideally we want to analyse the family samples in one common experiment. The prenatal diagnosis should be performed in a manner where the findings are confirmed by two different methods. At the same time the protocol used should allow for the exclusion of maternal contamination of the sample used as well as confirmation of paternity. In order to overcome the limitation arising from extracted DNA quality and quantity, we have developed and validated a multiplex HRM and STR analysis protocol, which allows us to analyse consistently and with high specificity, samples from variable sources extracted in different ways, in the same experiment. Here we report, the development and retrospective evaluation of the validity, diagnostic value and accuracy of a novel multiplex haplotyping and HRM method for CF prenatal diagnosis.

2. Materials and methods

2.1. Study materials

Peripheral blood samples were collected in EDTA containing tubes from 20 CF carrier couples, who were referred for prenatal diagnosis (PND) to DMG (genotype combinations are shown in Table 1) over a period of 10 years (2002–2012).

DNA was extracted from peripheral blood samples using either Qiagen’s QIAamp DNA Blood Mini Kit or MagAttract DNA Blood M48 Midi Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. Manual Salting Out method [10] was used to extract DNA from blood samples which were collected in 2003. Foetal samples were received at the DMG following standard chorionic villus sampling (CVS) at 12–14 weeks of gestation or amniocentesis at 15–17 weeks of gestation. DNA from CVS and AF samples was extracted by following Qiagen’s QIAamp DNA Blood Mini Kit protocol for tissue extraction. In some cases DNA samples corresponding to foetal tissue or cells were sent directly to the DMG for testing (Table 1). All the foetal DNA samples from past PND cases were kept under –80 °C. PND cases included in the study were analysed retrospectively. Initially, diagnosis was achieved through standard validated laboratory procedures involving DGGE analysis.
Diagnosis was subsequently confirmed by bi-directional sequencing and parallel haplotype analysis as previously described [11].

3.1. 1st round PCR reaction

A first 11-plex PCR reaction was optimized to simultaneously amplify 9 CF exon targets (bearing the most common mutations found in the Greek population) and 2 intragenic STR markers labelled with FAM/TAMRA to facilitate subsequent fragment analysis in an automatic sequencer (Supplementary data Table 1). The 11-plex PCR allows us to analyse simultaneously PND cases from families bearing different mutations. Primers for HRM were obtained from Montgomery et al. [8] (Supplementary data Table 1). Each 1st round of 20 μl multiplex PCR was performed using Multiplex HotStart Taq (Qiagen, GmbH Germany) according to the manufacturer’s instruction, adding CF exon primers and IVS8 at a final concentration of 0.15 μM and D7S677 at 0.2 μM using a touchdown PCR protocol ranging from 60 °C to 54 °C (PCR and cycling conditions are shown in Supplementary data Tables 2 & 3).

3.2. 2nd round PCR

After the first touchdown PCR, a second round “nested” PCR reaction was performed individually for each exon using Idaho’s LightScanner® Master Mix (Idaho Technologies) with the same exon primers as used in the 1st round PCR. The PCR products of the 1st round PCR reaction were diluted by 1/1000 (1 μl PCR product in 999 μl of DNase, RNase free ddH2O) and 2 μl of the diluted PCR product was used as a template for the “nested” reactions. The total volume of the 2nd round PCR reaction was 10 μl according to the LightScanner® Master Mix instructions and was set up in Qiagen’s PCR robotic system QIAgility (Qiagen) (Supplementary data Tables 5 & 6). The reaction was performed in Bio-Rad (Bio-Rad Laboratories California USA) compatible 96-well plates using the accompanying sealing films. 2nd round PCR was overlaid with 25 μl mineral oil (Sigma-Aldrich Corporation, St. Louis, MO). Before and after PCR cycling the plates were centrifuged at 3000 rpm for 2 min.

Exon 11 of CFTR gene (legacy exon 10) (CFTR reference sequence NM_000492.3 and NG_016465.1) carries p.Phe508del (c.1521_1523delCTT) which is the most common mutation occurring in CF patients and is very often found in a homozygous state. Exon 11 also includes a lot of non-disease causing polymorphisms which in combination with p.Phe508del can result in misinterpretation. For this reason, in order to detect it specifically, in the second PCR reaction we also used an unlabelled probe genotyping assay as previously described [12]. The conditions and cycling for the asymmetric 2nd round PCR for exon 11 are shown in Supplementary data Tables 6 & 7.

4. Melting analysis

After the 2nd round PCR reaction the products were imaged in a 96-well LightScanner (Idaho Technologies). PCR products were melted by increasing the temperature from 60 to 98 °C at a programmed rate of 0.1 °C/s with exposure set to automatic. When the probe was included in the plate the starting melting temperature was set at 50 °C. Melting curves were analysed with commercial LightScanner software.

4.1. STR analysis

Haplotype scoring, through IVS8CA and D7S677 fragment analysis, was facilitated by loading 1st round PCR products on an automatic sequencer (MegaBACE 1000 capillary sequencer, Amersham Pharmacia Biotech), following heat denaturation at 80 °C for 2 min. STR analysis is used to monitor maternal contamination and to confirm paternity in the analysed PND
samples. Linkage analysis was enabled in cases where DNA from an affected/unaffected sibling or the couple’s previously terminated foetus, was available for simultaneous analysis.

4.2. Generic HRM PND protocol diagnostic performance analysis

To assess the diagnostic performance of the HRM PND protocol, the sensitivity (Se), specificity (Sp), the false negative (FN) and false positive (FP) rates were calculated through the construction of Table 2. Genotypes obtained in the CVS and AF samples through the application of standard and validated laboratory diagnostic techniques (DGGE and sequencing) were defined as the ‘true genotypes’ and the genotypes achieved in the same samples by the generic multiplex HRM PND protocol were defined as the ‘test genotypes’. To facilitate and simplify parameter calculations, sample genotypes were converted into two categories: affected and unaffected. This step was taken because AF and CVS genotypes can potentially correspond to four categories: (i) wild type, (ii) heterozygous for the maternal mutation, (iii) heterozygous for the paternal mutation, and (iv) double heterozygote or homozygote (in cases where the parents carry the same mutation). Hence, affected genotypes correspond to double heterozygotes or homozygotes for a mutation and unaffected genotypes correspond to wild type and heterozygotes.

5. Results

5.1. 1st round and 2nd round PCR amplification and genotyping efficiency

Using this generic protocol we, retrospectively, tested 20 prenatal diagnosis cases for CFTR gene mutations (genotype combinations shown in Table 1) (a total of 76 samples). The number of samples tested includes the mother–father–embryo trio as well as previous children of the family, when this was available. All target sequences were successfully amplified in all samples analysed (76/76). Therefore the 1st round 11-plex PCR and 2nd round ‘nested’ PCR demonstrate an amplification efficiency of 100%.

Amplification of all targets was achieved in all study samples. This demonstrates the method’s robustness and flexibility, since DNA samples were extracted from different sources (amniotic fluid cells, chorionic villus and peripheral blood) by application of different extraction methods (automatic: Qiagen’s MagAttract DNA Blood Kit and manual: Qiagen’s QIAamp DNA Blood Mini Kit as well as Salting Out method) and were kept at −80 °C. Amplification was also achieved in already extracted DNA samples sent to our laboratory for analysis, without prior knowledge of the methodology used for extraction. Supplementary data Figs. 1 to 9 show the HRM results for each exon and Supplementary data Fig. 10 shows the haplotype analysis of one of the PND cases.

5.2. Diagnostic efficiency of the generic HRM PND method

The HRM PND method is an efficient, robust diagnostic tool as shown by the calculated diagnostic efficiency parameters. The method exhibited a sensitivity and specificity of 100% with FP and FN rates of 0% in genotyping a wide spectrum of mutations. The results show that the method is very sensitive and specific in genotyping archived samples from variable sources and different extraction methods.

The samples tested, using this protocol harboured mutations in nine different exons, most commonly identified in CF patients of Greek origin. The protocol allows the simultaneous amplification of different exons as well as two intragenic markers, for haplotype analysis. It is very important to stress that the multiplexing of this protocol allows all the PND cases in the laboratory to be analysed in a single experiment without worrying about the genotype combinations that each family carries.

6. Discussion

Since the implementation of a carrier screening programme for thalassemias in Greece, cystic fibrosis became the most common autosomal recessive disorder affecting young children. Therefore it is extremely important to be able to offer accurate carrier screening and prenatal diagnosis services. These services should also be offered in a cost effective way since the national health system cannot cover the expenses of a generalised screening programme. The increasing awareness for CF testing, has led to an increase of the samples being analysed. In order to compensate for this increase we had to update our testing methodologies and switch from DGGE analysis, which is labour intensive and does not allow large scale analysis, to HRM. HRM is a cost effective and robust method, showing the same sensitivity and specificity to DGGE. The most important advantage lies in the ability to perform large scale analysis in minimum time. For example in each DGGE gel we can load 20 samples and the running time is approximately 16 h, whereas for HRM each plate, containing 96 samples, can be analysed in 10 min. The timely molecular analysis is a key advantage, as in some cases the samples being processed derive from couples in the second trimester of pregnancy, mostly presenting with foetal echogenic bowel findings. Given the high molecular heterogeneity of the Greek population, these couples are screened for the entire CFTR coding sequence and if both parents are found carriers, of CF causing mutations, prenatal diagnosis is offered.

<table>
<thead>
<tr>
<th>Test genotypes</th>
<th>Affected (true positive)</th>
<th>Unaffected (false negative)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Affected</td>
<td>a</td>
<td>b (false positive)</td>
<td>a + b</td>
</tr>
<tr>
<td>Unaffected</td>
<td>c (false negative)</td>
<td>d (true negative)</td>
<td>c + d</td>
</tr>
<tr>
<td>Total</td>
<td>a + c</td>
<td>b + d</td>
<td>N</td>
</tr>
</tbody>
</table>

Sensitivity (Se); proportion a / (a + c) negative predictive value; proportion d / (c + d).
Specificity (Sp); proportion d / (b + d) positive predictive value; proportion a / (a + b).
False negative (FN); proportion c / (a + c).
False positive (FP); proportion b / (b + d).
Since DGGE analysis is no longer performed in our laboratory and each analysis, in a diagnostic setting, should be confirmed by two methodologies there was a great need to develop a novel protocol that allows the use of HRM technology in prenatal diagnosis.

One major drawback of HRM method is that it requires the use of the same DNA extraction method and purification. The use of samples from different sources, different extraction protocols or poor quality DNA in the same experiment is prohibitive. It is common practice, in order to successfully perform an HRM experiment, to analyse at least eight samples in the same batch. To do so, all the samples included in the experiment should derive from the same source and be processed the same way. This limitation poses a serious problem when working in a diagnostic laboratory, where the samples, sometimes, arrive from other laboratories as already isolated DNA or are from different biological sources.

The protocol we have developed allows us to analyse consistently and with high specificity, samples from variable sources, extracted in different ways emphasizing its application in prenatal diagnosis. The suggested method eliminates all possible differences between the samples allowing the accurate scanning with minimum labour and cost. Additionally the method allows the use of simple unlabelled probes in the second PCR reaction, which leads to specific mutation detection (genotyping). Fig. 1 presents a flow chart of the protocol.

The major advantage of this protocol is the simultaneous amplification of all CF regions of interest, for the specific couple, as well as the inclusion of intragenic polymorphic markers in the first PCR reaction. In this way we can perform direct prenatal diagnosis by mutation detection and indirect diagnosis, if the couple already has an affected offspring or biological material from a previous terminated affected pregnancy, by haplotype analysis within restrictive time limits. The diagnosis can be completed within one working day, alleviating the parental anxiety. After the second PCR reaction, the samples can be retrieved and sequenced with no need for further treatment, since the LCGreen Plus dye does not interfere with sequencing dyes. During the development and validation of this protocol we have included all different possible combinations of CFTR exons with no observed failure. The results presented in this paper however, refer to the exons most commonly analysed in our laboratory. Although HRM should be used for genotyping only when a probe is included in the reaction, in this protocol the use of a probe is not essential as we know in advance the parental genotypes. However if the expected genotypes are unknown this method can only be used as a scanning method and further genotyping is required.

This generic protocol for prenatal diagnosis of cystic fibrosis using HRM, has proven to be 100% efficient and specific, with no incidence of misdiagnosis. The protocol has been validated with retrospective analysis of 20 prenatal diagnosis cases, and is now routinely used in our laboratory. It is our belief that this protocol can be easily applied for other molecular analyses.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jcf.2014.04.002.

References


