Prenatal diagnosis of the thalassaemia syndromes by rapid DNA analytical methods

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Each couple requesting prenatal diagnosis was counselled either before initiation of pregnancy or as early as possible in the pregnancy, at which time blood samples were taken to confirm the haematological diagnosis and as a source of DNA for mutation analysis; the latter was essential before proceeding to fetal DNA analysis. Referral to a gynaeco-logist determined an appropriate date for fetal sample biopsy.

**Fetal sample preparation**

Fetal sampling protocols included the biopsy of chorionic villi (CVS; trophoblast) at 10–12 weeks of pregnancy or amniocentesis from ~14–20 weeks.

On receipt of a trophoblast sample, the villi were viewed under a microscope (×10 magnification) and carefully separated from any tissue that was of doubtful origin, placed in 1 ml of TE buffer (10 mM Tris, pH7; 1 mM EDTA) in sterile 1.5 ml Eppendorf tubes and washed twice prior to further processing. Amniotic cells were processed directly without culturing. The amniotic fluid samples (usually not more than 15 ml total volume) were transferred to sterile tubes and centrifuged to collect the cell pellet at 2500 g for 15 min in a bench centrifuge. The cell pellet was then transferred to 1.5 ml sterile eppendorf tubes and washed twice with TE before further processing. The fetal samples were either subjected to extraction with phenol–chloroform using a standard protocol (Old and Higgs, 1982) scaled down to micro volumes, or lysed by boiling for 2 min in 0.1 M NaOH/2 M NaCl (Kazazian, 1989).

**Mutation analysis**

The DNA diagnostic methods applied included those that directly characterize pathological mutations and those for indirect genotyping. In all prenatal samples, parental mutations were characterized prior to fetal DNA analysis and all fetal samples were subsequently assayed alongside the parental samples as controls.

**Denaturing gradient gel electrophoresis**

For the localization of β-thalassaemia mutations within the β-globin gene, several areas of the gene were independently amplified by PCR (Figure 1) and subsequently analysed by DGGE based on the protocol previously described (Losekoot et al., 1990), with the exception that gels were run at 200 V, 60 mA, for 5 h. The DGGE duplex patterns observed in parental samples directed the choice of method for direct mutation identification. The DGGE pattern, once confirmed to be associated with a particular pathological mutation in the parents, was subsequently assessed in the fetal sample.

**Amplification refractory mutation system**

The ARMS PCR method was applied for the detection of the nine most common Greek β-thalassaemia mutations (Old et al., 1990). Some modifications of the protocol were required in order to achieve allele-specific amplification for the mutant ARMS primers. These included titration of magnesium chloride concentrations and the quantities of primers (see Results). All samples under investigation were analysed simultaneously alongside positive and negative controls for the particular mutation. Following the PCR reaction, genotype results were ascertained by analysing the amplification products by agarose gel electrophoresis as described (Old et al., 1990). Fetal samples were always analysed alongside the parental DNA samples.

**Restriction enzyme analysis for mutation detection and diallelic β-globin gene cluster polymorphisms**

Amongst the samples assessed for prenatal diagnosis, eight mutations (Table I) were conveniently characterized by the loss or gain of restriction enzyme cutting sites (Kazazian, 1989).

The appropriate area of the gene was amplified using PCR (Figure 1), and subsequently subjected to restriction enzyme digestion and sizing of products by agarose gel electrophoresis. Results were always assessed in comparison with positive and negative controls for the mutation analysed simultaneously.

Restriction enzyme digestion analysis of the β-gene cluster diallelic polymorphic sites (Orkin et al., 1982) was based on previously described PCR protocols (Sutton et al., 1989; Old et al., 1990).

**Allele-specific oligonucleotide (ASO) hybridization**

Radioactively labelled allele-specific oligonucleotides (ASOs) were hybridized to α- or β-globin gene PCR products immobilized on Nylon filters (Amersham Life Science) by Southern blot alkali transfer as previously described (Traeger-Synodinos et al., 1993; Tzetis et al., 1994). Positive hybridization was detected by autoradiography under standard conditions and results were always judged in comparison with positive and negative controls for the mutation.

**PCR detection of deletions (‘gap’ PCR)**

Amongst the prenatal samples, it was necessary to investigate the presence of two δβ-thalassaemia deletions. The Sicilian-δβ, and Corfu-δβ, deletions were detected by previously described methods (Galanello et al., 1990; Craig et al., 1994).

**Assessment of maternal DNA contamination**

Haplotyping of the variable number of tandem repeat (VNTR) region 3’ to the ApoB gene on chromosome 2 was achieved by a PCR reaction followed by sizing of PCR products using agarose gel electrophoresis (Decorte et al., 1990). An alternative protocol included haplotyping of microsatellite sites in the CFTR gene (Morral and Estivill, 1992). In all prenatal samples, parental and fetal samples were haplotyped simultaneously to exclude maternal contamination (and non-paternity).

**Results**

A total of 105 trophoblast and 45 amniotic fluid cell samples were received for diagnosis. All fetal genotype results were confirmed by at least two independent analytical methods. The 145 pregnancies (148 fetal samples) at risk for β-haemoglobinopathies and thalassaemias, involved the analysis of 16 different pathological mutations (Table I). For nine pregnancies the pathological mutation in one parent remained uncharacterized. Linkage analysis was possible in two of these and seven cases were based on 50% diagnosis.

In addition, two prenatal diagnoses for non-deletional α-thalassaemia were requested, which involved the investigation of two non-deletion α-thalassaemia mutations (Table I). None of these diagnoses was only 50% informative.

Fetal DNA analysis for the 140 fully informative fetal samples diagnosed 33 normal, 77 heterozygote and 30 affected fetuses. Of the eight samples that were 50% informative, five were negative for the known mutation and three were positive.

For the preparation of fetal samples, the phenol extraction method was initially used, but as experience in handling the samples increased, the more rapid alkali lysis protocol became the preferred method. In all, 53 of the 105 CVS samples, and 20 of the 45 amniotic cell samples were processed by alkali lysis.

Prior to diagnosis of fetal samples, the mutations in the parents had to be characterized. Based upon the haematological phenotype of each individual (β-thalassaemia, δβ-thalassaemia, haemoglobinopathy, etc), appropriate techniques were selected from amongst the five PCR-based methods
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Figure 1. The PCR amplification of areas of the β-globin gene for denaturing gradient gel electrophoresis (DGGE), restriction endonuclease and allele-specific oligonucleotide (ASO) hybridization analyses. Black boxes are exons, white boxes are introns. 5'UTR and 3'UTR – 5' and 3' untranslated region. The location of each primer is indicated by the vertical lines and their direction by the arrows. The sequences of the primers are as follows:

A(GC): 5'-GTACGGCTGTCATACCTTAGACCTCA-3'
B: 5'-CAACCTCACTCCACGACTCC-3'
C: 5'-GGTGACCTGGAAGTT-3'
D(GC): 5'-AGTGAGCTGCACTGTGACAAGCTGCA-3'
E(GC): 5'-AGTGAGCTGCACTGTGACAAGCTGCA-3'
F: 5'-AAACGATCTGGACCTTCCA-3'
G: 5'-GTGTACACATTGACCAAA-3'
H: 5'-AGCAGACAGGACGACGTG-3'
I: 5'-ACCGTCCGCTGTCTGCT-3'
J: 5'-AATGACCTGACCTTCACA-3'

(GC) indicates that a 45 bp GC-clamp was attached at the 5' end of the primer for DGGE analysis.

Table I. Globin gene mutations in parents of 150 prenatal samples: approximate frequency of mutation and methods of detection. Figures in parentheses are percentages

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Approximate allele percentage among homozygotes</th>
<th>No. alleles in prenatal families</th>
<th>Detection methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-globin gene</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β+1VSI-110 (G&gt;A)</td>
<td>40</td>
<td>99 (33.5)</td>
<td>ARMS, DGGE</td>
</tr>
<tr>
<td>β0codon 39(C&gt;T)</td>
<td>16</td>
<td>46 (15.5)</td>
<td>ARMS, DGGE</td>
</tr>
<tr>
<td>β0IVSI-1 (G&gt;A)</td>
<td>12</td>
<td>25 (8.5)</td>
<td>ARMS, DGGE</td>
</tr>
<tr>
<td>β+1IVSI-6 (T&gt;C)</td>
<td>7</td>
<td>23 (7.7)</td>
<td>ARMS, DGGE</td>
</tr>
<tr>
<td>β+1IVSI-745 (C&gt;G)</td>
<td>6</td>
<td>13 (4.4)</td>
<td>ARMS, DGGE, gains Rsal site</td>
</tr>
<tr>
<td>β0codon 6 (-A)</td>
<td>2.5</td>
<td>4 (1.4)</td>
<td>ARMS, DGGE, loses Ddel site</td>
</tr>
<tr>
<td>β0IVSI-1 (-AA)</td>
<td>2</td>
<td>9 (3.0)</td>
<td>ARMS, DGGE, loses Hphl site</td>
</tr>
<tr>
<td>β+87 (C&gt;G)</td>
<td>1.5</td>
<td>8 (2.7)</td>
<td>ARMS, DGGE</td>
</tr>
<tr>
<td>β0codon 5 (-CT)</td>
<td>1</td>
<td>2 (0.7)</td>
<td>ARMS, DGGE, loses Ddel site</td>
</tr>
<tr>
<td>HbS</td>
<td>10</td>
<td>34 (11.5)</td>
<td>DGGE, loses Ddel site</td>
</tr>
<tr>
<td>δβCorfu plus</td>
<td></td>
<td></td>
<td>'gap' PCR (for the deletion)</td>
</tr>
<tr>
<td>JVI5(5&gt;G)</td>
<td>1</td>
<td>4 (1.4)</td>
<td>DGGE, gains EcRv site</td>
</tr>
<tr>
<td>β0codon 8 (-AA)</td>
<td>&lt;1</td>
<td>1 (0.3)</td>
<td>DGGE, ASO</td>
</tr>
<tr>
<td>HbO-Arabb</td>
<td>&lt;1</td>
<td>1 (0.3)</td>
<td>loses EcoR1 site</td>
</tr>
<tr>
<td>HbO-Punjabb</td>
<td>&lt;1</td>
<td>1 (0.3)</td>
<td>loses EcoR1 site</td>
</tr>
<tr>
<td>δβSicilian</td>
<td>&lt;1</td>
<td>4 (1.4)</td>
<td>'gap' PCR</td>
</tr>
<tr>
<td>β+101 (C&gt;T)</td>
<td>?</td>
<td>7 (2.4)</td>
<td>DGGE, ASO</td>
</tr>
</tbody>
</table>

Non-deletion α-thalassaemia

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Approximate allele percentage among homozygotes</th>
<th>No. alleles in prenatal families</th>
<th>Detection methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saudi polyA</td>
<td>2 (0.7)</td>
<td></td>
<td>ASO</td>
</tr>
<tr>
<td>Codon 29 (HbAgrino)</td>
<td>1 (0.3)</td>
<td></td>
<td>ASO</td>
</tr>
<tr>
<td>Uncharacterized β-thalassaemia</td>
<td>9 (3.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uncharacterized α-thalassaemia</td>
<td>1 (0.3)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ARMS = amplification refractory mutation system; DGGE = denaturing gradient gel electrophoresis; ASO = allele-specific oligonucleotide hybridization.

bParent was compound heterozygote for βIVSI-110 and HbO-Arab. The couple was counselled accordingly.

described in the methodology to genotype each parental, and subsequently each fetal sample.

DGGE was the primary method used for screening the β-globin gene in the heterozygous parents (Figure 2) to localize the pathological mutations. Most β-gene mutations are associated with distinct electrophoretic patterns, thus directing the choice of method for specifically characterizing the mutation. However, certain areas of the β-globin gene include polymorphic nucleotide changes which may confuse assessment of DGGE analysis and all results needed careful interpretation; a DGGE pattern alone was never considered a definitive diagnosis for a pathological mutation in parental samples.

DGGE analysis was successfully applied to genotype 104 fetal samples. All fetal genotype results obtained by DGGE
Following denaturing gradient gel electrophoresis (DGGE) analysis, the gel is stained with ethidium bromide and viewed with ultra UV. The analysis of part of the β-globin gene shows the following genotypes: Lanes 1, 4, 6, 8: no mutation; Lane 2: homozygote β+IVSI-6(T>C); Lane 3, 13: heterozygote β+IVSI-6(T>C); Lane 5, 9: heterozygote β+IVSI-110(G>A); Lane 7, 11: heterozygote β+IVSI-5(G>A); Lane 10: heterozygote codon39(C>T); Lane 12: heterozygote β0IVSI-1(G>A).

Table II. Optimized conditions for amplification refractory mutation system (ARMS) polymerase chain reaction (PCR) detection of common Greek β-thalassaemia mutations

<table>
<thead>
<tr>
<th>β-thalassaemia mutation</th>
<th>Concentrations of MgCl2(nM)</th>
<th>Concentration of primer (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β+IVSI-110(G&gt;A)</td>
<td>2.5</td>
<td>5</td>
</tr>
<tr>
<td>β0IVSI-1(G&gt;A)</td>
<td>2.0</td>
<td>5</td>
</tr>
<tr>
<td>β+IVSI-6(T&gt;C)</td>
<td>2.5</td>
<td>15</td>
</tr>
<tr>
<td>β0codon 39(C&gt;T)</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>β0codon 6(-A)</td>
<td>4.5</td>
<td>15</td>
</tr>
<tr>
<td>β0IVSI-1(G&gt;A)</td>
<td>2.0</td>
<td>5.0</td>
</tr>
<tr>
<td>β+IVSII-745(C&gt;G)</td>
<td>3.0</td>
<td>10</td>
</tr>
<tr>
<td>β+ minus 87(C&gt;G)</td>
<td>3.0</td>
<td>10</td>
</tr>
<tr>
<td>β0codon 5(-CT)</td>
<td>5.0</td>
<td>10</td>
</tr>
</tbody>
</table>

The recommended primer concentrations (Old et al., 1990) were modified to achieve mutation-specific amplification.

Restriction endonuclease (RE) analysis was applicable for the characterization of eight β-globin gene mutations observed in 62 of the pathological alleles in the parents (Table I). The presence or complete absence of a mutation was clearly distinguished by RE analysis in all cases. However, for the mutations β0 codon 6(-A), β0 codon 5(-CT) and HbS, which destroy a cutting site for the restriction enzyme Ddel, the definitive presence of a normal allele was not always clearly distinguished when genotyping the fetuses of parents that were carriers for the same mutation (five cases: four for HbS and one for β0 codon 5-CT) and an alternative method was applied.

The hybridization of radioactively labelled allele specific oligonucleotides was used to characterize at least one mutation in 22 of the prenatal samples. Five of these included genotypes with no alternative method for characterization (Table I) and
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Discussion

Until long-term curative therapy becomes a feasible option for treating genetic disorders, prenatal diagnosis for avoiding the birth of affected individuals is the main approach to controlling such diseases and has become an accepted procedure in most populations worldwide. In this study only one affected pregnancy was not terminated, and even in the three cases based on 50% informativity which were all positive for the known mutation, the pregnancies were terminated.

In Greece, the thalassaemia syndromes and haemoglobinopathies are extremely heterogeneous both at the molecular and consequently phenotypic level. To offer a prenatal diagnostic service through DNA analysis it is essential that parents have correct haematological diagnosis (hence the necessity of close collaboration with a specialized haematology laboratory), and that the DNA diagnostic laboratory has several standardized analytical techniques.

The aim of prenatal diagnosis is to provide an accurate and rapid result as early in the pregnancy as possible. Until preimplantation diagnostic protocols have been optimized (Thornhill et al., 1994; Findlay et al., 1995), a prerequisite involves obtaining fetal material promptly and safely. With present protocols, trophoblast samples can be obtained at 8–10 weeks of gestation (Froster and Jackson, 1996). If CVS sampling is not an option for the pregnancy in question, then we have observed that satisfactory quantities of amniotic fluid cells for PCR analysis are obtained through amniocentesis from as early as 14 weeks of pregnancy.

For the preparation of genetic material from fetal samples both the phenol extraction method and the alkali lysis protocol were effective but the latter was simpler and faster, preparing a sample ready for PCR set-up within 20 min.

The DNA analytical methods chosen in our laboratory included DGGE, ARMS PCR, restriction endonuclease analysis, ASO hybridization and ‘gap’ PCR.

DGGE was the primary method of choice, localizing the pathological mutation within the β-globin gene in β-thalassaemia heterozygotes and genotyping fetal samples once parental mutations were established. However, DGGE is technically very challenging. It requires sophisticated equipment and is subject to many causes for methodological failure, including the batch quality of all chemical components of the gels, buffer, PCR primers and gel running conditions. This must be taken into consideration when selecting protocols for a routine diagnostic service.

ARMS PCR was the other primary method applied for analysing β-thalassaemia mutations in our laboratory. It is a direct mutation detection method and is extremely rapid, producing results within a few hours of PCR set-up by simply analysing the amplification products using agarose gel electrophoresis. It proved to be extremely reliable in almost all prenatal cases, although we found it difficult to standardize for differentiating between heterozygous or homozygous genotypes for a single pathological mutation.

Restriction endonuclease analysis was used as a secondary method. For direct mutation analysis it is simple but limited to specific mutations. Linkage analysis using diallelic polymorphic restriction sites also has limited application, since DNA genotyping in additional family members is necessary in order to establish linkage and several polymorphic sites may have to be analysed to find those that are informative.

The hybridization of radioactively labelled allele-specific oligonucleotides (ASOs) was standardized for the characterization of all pathological α- and β-globin gene mutations described in the Greek population. The method was found to be simple and reliable when results were judged in comparison with known positive and negative controls. However, because the ASO probes were radioactively labelled in the protocol established in our unit, it was only applied when all other methods had failed or were unavailable for a particular mutation under investigation, thus avoiding the use of radioactivity which is hazardous for the operator and expensive.

The principle of ‘gap’ PCR is that PCR primers located far apart in native DNA are unable to direct amplification unless an intervening genomic DNA deletion brings them in to closer proximity. Although the PCR based detection of deletions was only applicable in a few prenatal samples, it was consistently reliable in all cases and provided results within a few hours.

Finally, the extreme sensitivity of PCR introduces the possibility of contamination and false results. Even when general precautions are taken to avoid contamination from sources in the laboratory, minimal quantities of maternal cells in the fetal sample could produce a spurious diagnosis. To exclude this possibility, polymorphic regions of the genome were analysed in both parents and fetal samples, particularly in those cases in which the fetus was identified to carry at least the mother’s mutation. The sizing of the VNTR 3’ to the ApoB gene involved a simple protocol but was not always informative and it was necessary to have an alternative protocol established in the laboratory.

In conclusion, substantial advances have been made in the
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last few years in DNA diagnostics, due to the development of numerous rapid analytical methods based on PCR. Although most methods give reliable results, some are technically sophisticated or difficult to standardize and all are subject to periodic failure. Furthermore, few have been developed for high throughput DNA diagnostics. To offer a rapid and accurate DNA diagnostic service for a molecularly heterogeneous disease, our results illustrate that the availability of several alternative standardized methods is essential. On this basis our laboratory can offer a prenatal diagnosis within a few working days following receipt of the fetal sample; thus from a trophoblast biopsy the parents may have a result early within the second trimester of pregnancy.

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