Rapid detection of medium chain acyl-CoA dehydrogenase gene mutations by non-radioactive, single strand conformation polymorphism minigels

Achille Iolascon, Teresa Parrella, Silverio Perrotta, Ornella Guardamagna, Paul M Coates, Maria Sartore, Saul Surrey, Paolo Fortina

Abstract
Medium chain acyl-CoA dehydrogenase (MCAD) deficiency is a common inherited metabolic disorder affecting fatty acid β oxidation. Identification of carriers is important since the disease can be fatal and is readily treatable once diagnosed. Twelve molecular defects have been identified in the MCAD gene; however, a single highly prevalent mutation, A985G, accounts for >90% of mutant alleles in the white population. In order to facilitate the molecular diagnosis of MCAD deficiency, oligonucleotide primers were designed to amplify the exon regions encompassing the 12 mutations enzymatically, and PCR products were then screened with a single strand conformation polymorphism (SSCP) based method. Minigels were used allowing much faster run times, and silver staining was used after gel electrophoresis to eliminate the need for radioisotopic labelling strategies. Our non-radioactive, minigel SSCP approach showed that normals can be readily distinguished from heterozygotes and homozygotes for all three of the 12 known MCAD mutations which were detected in our sampling of 48 persons. In addition, each band pattern is characteristic for a specific mutation, including those mapping in the same PCR product like A985G and T1124C. When necessary, the molecular defect was confirmed using either restriction enzyme digestion of PCR products or by direct DNA sequence analysis or both. This rapid, non-radioactive approach can become routine for molecular diagnosis of MCAD deficiency and other genetic disorders.

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for detection of base changes in PCR products, and has been recently used to distinguish DR4 alleles for determination of HLA compatibility.21 The technique is based on the principle that under non-denaturing conditions the electrophoretic mobility of single stranded nucleic acid depends not only on size but also DNA sequence.22 We present important modifications to the original SSCP protocol which facilitate rapid non-radioactive delineation of mutations causing disease. Our approach involves the use of minigels which also allows much faster run times, and silver nitrate staining for single stranded DNA detection which eliminates the need for radioisotopic labelling strategies. Pharmacia LKB Biotechnology (Alameda, CA) recently introduced a pre-cast gel system which uses a similar approach to mutation detection. Our protocol including PCR steps takes about four to six hours.

Forty-eight samples from white persons of northern Europe ancestry were referred to the Children’s Hospital of Philadelphia for determination of MCAD deficiency. The majority of those tested were sibs or parents of children diagnosed with sudden infant death syndrome, from families with a history of MCAD disease, or had atypical profiles for excreted organic acids and enzymatic assays. Genomic DNA was extracted from peripheral blood leucocytes using previously described protocols.24 Four segments encompassing exons 3 and 4, exon 6, exon 9, and exon 11 of the human MCAD gene were amplified using a DNA thermocycler as previously described.25 Oligonucleotide sequences with MCAD gene coordinates and PCR product sizes are listed in the table. Amplifications were performed for 30 cycles in 100 μl of reaction containing 300 ng of genomic DNA, 6.7 mmol/l MgCl₂, 16.6 mmol/l (NH₄)₂SO₄, 5 mmol/l βME, 6.8 mmol/l EDTA, 67 mmol/l Tris Cl pH 8.8, 200 μmol/l dNTP, 0.15 μmol/l primers, and 2.5 U AmpliTaq polymerase (Perkin Elmer Cetus, Norwalk, CT). Each cycle included the following denaturation, annealing, and Taq polymerase extension conditions: 94°C for 45 seconds, 60°C for 30 seconds, and 72°C for 45 seconds for exon 3, 4, and 9 amplifications; 95°C for one minute, 50°C for one minute, and 72°C for 1.5 minutes for exon 6; and 94°C for two minutes, 56°C for one minute and 30 seconds, 72°C for two minutes for exon 11. Up to a maximum of 16 PCR products (8 × 2 minigels) can be electrophoresed on a non-denaturing minigel (7.2 cm × 10.2 cm) (Bio-Rad Laboratories, Melville, NY) containing 10–12% (w/v) acrylamide (acrylamide/bis 38:1 (w/w)) and 5–10% (v/v) glycerol. PCR product (2 μl) is mixed with 10 μl of denaturing solution (95% (v/v) formamide, 0.05% (w/v) each of bromophenol blue and xylene-cyanol in 20 mmol/l EDTA), samples are heated at 96°C for five minutes, cooled in a 4°C ice bath, and then electrophoresed in 1 × TBE buffer at 4°C using 120–150 volts for two to four hours. Visualization of single stranded DNA was performed by standard silver nitrate staining (Bio-Rad Laboratories).

SSCP patterns for the different MCAD mutations are shown in the figure. All samples showing normal SSCP patterns were subjected to automated DNA sequence analysis and shown to have normal sequence in the PCR products of exons 3, 4, 9, and 11 (data not shown). Two of the eight known mutations in exon 11 were detected in our samples using SSCP (lanes 2 and 3). The patterns for normal (N), A985G, and T1124C mutations were clearly different. Assignment of N/N (figure, lane 1), A985G/A984G (lane 2), A985/T1124C (lane 3), and A985G/N (genotypes (lane 5) in the 249 bp PCR product from exon 11 was confirmed either by digestion with NcoI for A985G or AccI for T1124C, as described previously.20 The only known mutation in exon 6 was also readily detected in our samples using SSCP (lanes 7 and 8). Patterns for normals (lane 6) and G447A (lane 8) were clearly resolved, and assignment of the G447A/N genotype (lane 8) in the 81 bp PCR product from exon 6 was confirmed by digestion with MseI as described previously. These results corroborated by direct DNA sequence analysis of the PCR products suggest that this SSCP protocol has not missed detection of any other disease causing mutation in these MCAD gene exon regions in the samples we analysed. Results of our analysis of the 48 samples showed the following: 29 were negative for any of the 12 known mutations, and there were 11 heterozygotes and six homozygotes for A985G, one heterozygote for G447A, one compound heterozygote for A985G/ T1124C, and one for A985G/G447A. Further studies are in progress to analyse additional MCAD gene regions by SSCP for potential disease causing mutations in the remaining samples.

Discussion
This non-radioactive minigel SSCP approach can readily distinguish at least three of the 12 known MCAD disease causing mutations and may in fact distinguish the remaining known MCAD mutations. Additional samples containing the remaining nine mutations are now required to test this. In addition, each band pattern is characteristic for a specific mutation, including those mapping in the same PCR

**Oligonucleotides used for genomic DNA amplification and DNA sequence analysis**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5'→3'</th>
<th>Exon</th>
<th>PCR product (bp)</th>
<th>Coordinate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCAD-1-F</td>
<td>AGTTCTACGCGAACAAGAGA</td>
<td>3 and 4</td>
<td>202</td>
<td>119-136</td>
</tr>
<tr>
<td>MCAD-2-F</td>
<td>TCCACAGGCTCTTCTTAAATT</td>
<td>5</td>
<td>222</td>
<td>207-249</td>
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<tr>
<td>MCAD-3-F</td>
<td>CAAAGTCTATTATTTATT</td>
<td>6</td>
<td>201</td>
<td>308-405</td>
</tr>
<tr>
<td>MCAD-4-F</td>
<td>ACCATCAATGTCGCTTTC</td>
<td>4</td>
<td>241</td>
<td>451-490</td>
</tr>
<tr>
<td>MCAD-5-F</td>
<td>GAAATTACAAGCTGCA</td>
<td>9</td>
<td>141</td>
<td>709-725</td>
</tr>
<tr>
<td>MCAD-6-F</td>
<td>TACAGGTCAGTTCTTATG</td>
<td>11</td>
<td>249</td>
<td>822-849</td>
</tr>
<tr>
<td>MCAD-7-F</td>
<td>TACAGGCTACATTTATG</td>
<td>11</td>
<td>249</td>
<td>946-966</td>
</tr>
<tr>
<td>MCAD-8-R</td>
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<td>9</td>
<td>249</td>
<td>1174-1194</td>
</tr>
</tbody>
</table>

* Coordinates refer to published sequence of the MCAD gene.24
Rapid detection of medium chain acyl-CoA dehydrogenase gene mutations

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20 Yokota I, Indo Y, Coates PM, et al. Molecular basis of medium chain acyl-coenzyme A dehydrogenase deficiency. An A to G transition at position 985 that causes a lysine-304 to glutamate substitution in the mature protein is the
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