Preimplantation genetic diagnosis for medium-chain acyl-CoA dehydrogenase (MCAD) deficiency


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Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency is the most common defect in fatty acid oxidation. The disease is inherited in an autosomal recessive fashion (carrier frequency around 1 in 70) and probably affects as many as 1 in 10,000 newborns. Affected children usually present within the two first years of life with recurrent episodes of hypoketotic hypoglycaemia and lethargy leading to death in ~25% of the cases. One mutation (c985A→G) accounts for ~90% of the carrier chromosomes. We developed a preimplantation genetic diagnosis (PGD) strategy for MCAD for a couple who had already lost two affected children. When tested on heterozygous lymphoblasts, the amplification efficiency was 67 out of 71 (94%) and the allele dropout rate was 0 out of 67. The patient became pregnant after one PGD cycle during which two embryos were replaced. The twin pregnancy was checked by chorionic villus sampling (CVS) and was shown to be unaffected. The twins have been born and are healthy.

Key words: medium-chain acyl-CoA dehydrogenase deficiency/preimplantation genetic diagnosis/single-cell PCR

Introduction

Medium-chain acyl-CoA dehydrogenase (MCAD, MIM #201450) deficiency is one of the most common recessively-inherited defects in fatty acid oxidation, which takes place in the mitochondria. The incidence of the disease may be as high as 1 in 10,000 births in Northern Europe, with an estimated carrier frequency of the most frequent mutation (c985A→G) of between 1 in 50 (Finland) and 1 in 240 (Czech Republic) (Tanaka et al., 1997). Affected children usually present within the first two years of life during periods of fasting (e.g. during concurrent viral gastro-intestinal infection) with recurrent episodes of hypoglycaemia associated with very low or no ketone bodies in their urine, reflecting their inability to metabolize medium-chain fatty acids. They often present in a coma or with an altered level of consciousness and ~25% of affected children die during this initial presentation. Long-term follow-up of MCAD deficiency patients has shown significant morbidity, ranging from low achievement at school to severe handicap (epilepsy, hemiplegia and marked delay), amongst survivors of the initial episode (Wilcken et al., 1994). Therapeutic measures, e.g. appropriate diet (frequent feeding), vitamin and carnitine supplements can avoid the occurrence of these life-threatening episodes.

The enzyme deficiency responsible for the disease was characterized in 1983 by three groups (Divry et al., 1983; Rhead et al., 1983; Stanley et al., 1983) and the gene was cloned in 1987 (Kelly et al., 1987). Other authors (Matsubara et al., 1990; Gregersen et al., 1991) were able to show that the commonest mutation, found in ~90% of the carrier chromosomes, was an A→G transition at cDNA position 985, resulting in a change of amino acid residue 329 from lysine to glutamic acid (K329E). Until recently, 21 other and rarer mutations have been described (Andresen et al., 1997). In the first reports on the c985A→G mutation, a quick method to determine the presence of the mutation was described (Matsubara et al., 1990; Gregersen et al., 1991): using polymerase chain reaction (PCR) with a primer mismatched at the site of the mutation, a NcoI restriction site was introduced in the mutated sequence. We have used an adaptation of this strategy for the development of a PCR test at the single-cell level. This test was used for preimplantation genetic diagnosis (PGD) in a couple of which both partners carried the commonest c985A→G mutation and who had already lost two affected children.

Materials and methods

Patient description

Patients were a healthy, non-consanguineous couple. The wife was aged 31 and the husband 32 years at the time of treatment. The couple had lost two children from MCAD at 8 and 9 months of age presenting as sudden infant death syndrome (SIDS) after an episode of gastro-intestinal problems. The diagnosis of MCAD deficiency was made after the second child had died and the c985A→G mutation was found in peripheral blood DNA of both parents (heterozygotes) and the affected child (homozygote).

Sampling of single lymphoblasts

Lymphocytes from the mother were transformed with Epstein-Barr virus and cultured as described earlier (Ventura et al., 1988).
Lymphoblast colonies were collected and single lymphoblasts were put in 0.2 ml PCR tubes containing 2.5 µl alkaline lysis buffer (ALB; 200 mM NaOH, 50 mM dithiothreitol) as described previously (Sermon et al., 1998b). Per two lymphoblasts collected, one tube containing an aliquot from the last washing droplet in ALB served as a blank. Samples were kept at -80°C until further processing.

**Sampling of single blastomeres from research embryos**

Embryos from our intracytoplasmatic sperm injection (ICSI) programme which were unsuitable for transfer because they contained >50% fragments and/or irregular blastomeres or were unsuitable for cryopreservation because they contained >20% fragments were donated by the patients after giving informed consent and with the approval of the institutional ethical committee. The zona pellucida was removed using acidic Tyrode’s solution and single blastomeres were transferred to 0.2 ml PCR tubes containing 2.5 µl ALB as described previously (Sermon et al., 1998a). For each blastomere, one tube containing an aliquot of the last washing droplet served as a blank. Samples were kept at -80°C until further processing.

**ICSI procedure**

The ICSI procedure was performed as previously described (Van Steirteghem et al., 1998). Ovarian stimulation was carried out by a desensitising long protocol of the intranasally administered gonadotrophin-releasing hormone agonist, buserelin (Suprefact®; Hoechst, Brussels, Belgium) in association with 150 IU human menopausal gonadotrophin (HMG) daily (Humegon®; Organon, Oss, The Netherlands; or Pergonal®; Serono, Brussels, Belgium). Human chorionic gonadotrophin (HCG; 10 000 IU) was administered (Pregnyl®; Organon; Profasi, Serono) when at least nine follicles with a diameter of 17 mm were visualized on pelvic ultrasound scan (Vandervorst et al., 1998). Cumulus-oocyte-complexes (n = 14) were retrieved by vaginal ultrasound-guided puncture of the ovarian follicles, 36 h after HCG administration. Eight metaphase II oocytes were injected with a single sperm. The Petri dishes with the oocytes were incubated in 25 µl droplets of S1 medium (Scandinavian IVF) until after the biopsy in the morning of day 3 and in S2 medium (Scandinavian IVF) from then on. Five oocytes were normally fertilized and four embryos developed to the seven-cell stage or beyond.

**Embryo biopsy**

Embryo biopsy was carried out as previously described (Sermon et al., 1998a). Only embryos which had developed to the 7-cell stage and beyond were considered for biopsy, since our policy is to biopsy two blastomeres from each embryo in order to avoid or reduce the risk of misdiagnosis (Sermon et al., 1998b). Briefly, a hole was made in the zona pellucida using a stream of acidic Tyrode’s solution (Scandinavian IVF, Goteborg, Sweden), after which two blastomeres were removed by aspiration. Four embryos which had developed beyond the 7-cell stage were biopsied and eight blastomeres were obtained. Embryos nos. 1 and 2 were at the 8-cell stage, embryo no. 6 contained seven cells and embryo no. 8 contained nine cells. The blastomeres were checked for the presence of a nucleus, were washed three times in Mg²⁺ and Ca²⁺-free medium and were transferred to a PCR tube containing 2.5 µl ALB. Per single blastomere, a small aliquot of the last washing droplet was added to a separate PCR tube containing ALB. The blastomeres were kept at -80°C for 30 min before further processing.

**PCR procedure**

The following newly designed primer set was used, yielding a fragment of 186 bp: MCADK1 (forward primer): 5'-TGCTGGCTG-AATGCCCAGT-3' and MCADK2 (reverse primer, labelled with Cy5): 5'-CTGATTATATCCATGGCTC-3'. The bold C in the forward primer is the mismatched nucleotide which introduces a restriction site in the mutated allele. As the point mutation is immediately downstream of the forward primer, the mutant template will direct the insertion of G, rather than A, thus producing an NcoI restriction site (CCATGG). Therefore, a wild-type allele will remain uncut by NcoI, while a mutated allele will yield a fluorescently-labelled 170 bp fragment and an unlabelled 16 bp fragment.

A reaction mix containing 0.4 µmol/l of each primer, 200 µmol/l dNTP, 1× Expand High Fidelity Buffer (Roche, Brussels, Belgium), 20 mmol/l Tricine pH 4.95 for a total volume of 25 µl per sample was prepared and decontaminated with the restriction enzyme HaeIII by incubation at 37°C for at least 3 h, after which the restriction enzyme was inactivated by heating at 65°C for at least 20 min.

The single cells were lysed by incubating them at 65°C for 10 min in ALB. Expand High Fidelity polymerase (2.6 IU per sample) was added to the reaction mix, briefly mixed, and 22.5 µl of the reaction mix was added to each single cell sample. PCR was performed on a Perkin Elmer PCR System 24000 (Brussels, Belgium) using the following programme: 2 min denaturation at 95°C followed by 5 cycles of 15 s at 95°C, 30 s at 55°C and 45 s at 72°C followed by an extension for 7 min at 72°C.

The PCR reaction was followed by a digestion of 1 h at 37°C with 10 IU NcoI. Six µl of the restriction product was mixed with 6 µl loading buffer (5 mg/ml Dextran Blue 2000 in deionized formamide). Samples were loaded on a 6% sequencing gel (Life Technologies, Ghent, Belgium), and run on an ALFExpress Automated Sequencer (Amersham Pharmacia Biotech, Roosendaal, The Netherlands). The results were processed using the fragment analysis software provided by the manufacturer (Allele Links, Amersham Pharmacia Biotech).

**Results**

**Single lymphoblasts from the mother**

A total of 71 single lymphoblasts from the carrier mother were assayed in five different experiments. Out of 71 lymphoblasts, 67 showed amplification (94.4%) and one out of 35 blanks showed contamination (2.9%). None of the samples showed allele drop-out (ADO) after digestion with the NcoI restriction enzyme.

**Single blastomeres from spare embryos**

In all, 51 blastomeres from 15 embryos were analysed. Of these, 47 showed amplification (92.2%), while none of the 51 blanks showed contamination. No restriction was carried out on these samples as it was assumed that the probability that any of the embryos would be a carrier of the c985A→G mutation was low.

**PGD cycle**

All eight biopsied blastomeres showed amplification. Six single blastomeres from embryos 1, 2 and 3 showed a heterozygote restriction pattern and no ADO. Embryo 4 was homozygous normal (Figure 1). Embryos nos. 1 (a heterozygote 7-cell grade B at transfer) and no. 4 (a homozygous normal, 7-cell, grade B) were transferred in the evening of day three, resulting in an ongoing dizygotic twin pregnancy. Embryo no. 3 was cryopreserved in the morning of day 4 (8-cell, grade B). The
PGD for MCAD deficiency

Figure 1. Data from the ALFExpress gel electrophoresis of the PGD cycle. Lane 5 shows a 50–300 bp ladder. Amplified genomic DNA from a control (lane 2, 186 bp) and an affected person (lane 3, 170 bp) are shown. The results of embryo no. 1 are shown in lanes 6 and 7. This embryo was diagnosed as heterozygous for the mutations. The same results were obtained in embryos nos. 2 and 3 (lanes 8, 9, 10 and 11). In lanes 12 and 13, a homozygous normal embryo (no. 4) was diagnosed. Embryos nos. 1 and 4 were transferred to the mother.

result was confirmed in embryo no. 2, which was unsuitable for cryopreservation.
At 12 weeks gestation, chorionic villus sampling (CVS) was performed and the diagnosis was confirmed (one heterozygote and one homozygous normal twin). The twins (a girl and a boy) were born vaginally at 37 weeks gestation, weighing 2740 and 3010 g respectively; both had good Apgar scores. Both babies were seen at 2.5 months of age and were developing normally (weight between 25th and 50th percentile for both children, height third percentile for both children, head circumference between 25th and 50th percentile for both children), except that the girl had a congenital hip luxation for which she had to wear double diapers.

Discussion
MCAD deficiency, with its high carrier frequency in Northern Europe (carrier frequency for c985A→G of 1 in 77 in the Belgian population; Tanaka et al., 1997), is one of the most frequent autosomal recessive diseases in the Caucasian population. It is estimated that the condition is lethal in 25% of the affected infants if MCAD deficiency is not diagnosed in time, which is why it can be considered as a serious disease, although efficient treatment is now available. MCAD deficiency may also account for a small proportion of sudden infant death syndromes (SIDS), although in MCAD deficiency the infant often shows signs of the disease such as hypoglycaemia, altered level of consciousness and coma, and fatty infiltration can be found in the liver at post-mortem examination. These signs are absent in classic SIDS. Moreover, several retrospective studies and one prospective study were unable to demonstrate a higher incidence of MCAD deficiency in SIDS cases than in the general population (Matsubara et al., 1991; Miller et al., 1992; Arens et al., 1993; Penzien et al., 1994).

The severity of the disease, as well as its relatively high frequency and the fact that one mutation can be found in 90% of the carrier chromosomes, made it a good candidate for the development of PGD. In this case, the patient couple who requested PGD for MCAD deficiency had already lost two affected children and were both carriers of the c985A→G mutation.

The single-cell PCR assay that we present here shows high amplification efficiency on lymphoblasts (67/71, 94%) as well as on blastomeres (47/51 + 8/8 = 55/59; 93%). The use of fluorescent PCR, followed by analysis on an automated sequencer involves a low level of ADO [no ADO observed in 73 heterozygote cells (lymphoblasts and blastomeres) analysed], as was shown by previous authors (Findlay et al., 1996a,b; Sermon et al., 1998a). The accuracy of the test was confirmed after a successful PGD cycle, not only by re-analysis of the non-transferred embryo, but also by prenatal diagnosis of the ensuing pregnancy. Even so, since the occurrence of a misdiagnosis for myotonic dystrophy (Sermon et al., 1998b), our policy has been to biopsy two cells for diagnosis, even for a test as reliable and efficient as the one described here. Another type of PGD assay for MCAD caused by the c985A→G mutation was recently described (Iouliano et al., 2000). Here, the mutation is analysed concurrently with a linked polymorphism for which the family is informative, which increases the chance to detect ADO or contamination if it occurs. Using this type of test, it would probably not be necessary to biopsy two cells. Hence, we have recently decided that, from now on, all newly developed tests would be based on duplex PCR.

Finally, the fact that the two transferred embryos implanted and developed into two normal, healthy babies, suggests, as was already shown extensively on research embryos (Hardy et al., 1990) that biopsy of two blastomeres at the 7-cell stage or beyond, which we consider essential for accurate diagnosis, does not compromise further development of the embryo. Analysis of our other PGD cycles in which two blastomeres were biopsied per embryo confirms this observation on a larger scale (Van de Velde et al., 2000).

References


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