0031-3998/95/3705-0675$03.00/0
PEDIATRIC RESEARCH

Vol. 37, No. 5, 1995
Printed in U.S.A.

Medium Chain Acyl-CoA Dehydrogenase Deficiency in Pennsylvania: Neonatal Screening Shows High Incidence and Unexpected Mutation Frequencies

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ABSTRACT

Medium chain acyl-CoA dehydrogenase deficiency (MCAD) is a defect in the mitochondrial oxidation of fatty acids. The disorder typically presents with episodes of vomiting and hypoglycemia, sometimes with changes in mental status and hepatic failure. These Reye's-like features may culminate in coma and death. Stress, intercurrent illness, and reaction to childhood immunization have been shown to precipitate acute metabolic episodes in MCAD patients. All cases are caused by mutations of the single MCAD gene on chromosome 1. Most clinically ascertained cases are caused by an A985G transition in exon 11. Here we report the preliminary findings of MCAD patients detected prospectively through a supplemental newborn screening program in Pennsylvania using tandem mass spectrometry. From the first 80,371 newborns screened we prospectively found nine babies with MCAD (1/8930) plus two additional newborns screened because of a previously known family history. Molecular analysis showed 56% of the detected patients to be compound heterozygotes for the A985G and a second mutation. This is in contrast to clinical retrospective studies which have found only 20% to be compound heterozygotes. We have identified two of the other mutations including a novel mutation (DG91/C92, 6-bp deletion) in one of our patients by using single-stranded conformation polymorphism (SSCP) and sequence analysis of conformers. Our results confirm that MCAD is one of the more common inborn errors of metabolism. The different mutation frequencies observed between retrospective clinical studies and our prospective newborn screening study suggest that clinical ascertainment may lead to preferential identification of the A985G mutation. (***Pediatr Res* 37: 675–678, 1995**)

Abbreviations

MCAD, medium chain acyl-CoA dehydrogenase deficiency
SSCP, single-stranded conformation polymorphism
DG91/C92, deletion of glycine 91 and cysteine 92
PCR, polymerase chain reaction
MS/MS, tandem mass spectrometry

The first description of MCAD was made, in 1976, by Gregersen *et al.* (1), and the disorder was first characterized enzymatically by Kolvraa *et al.* (2). MCAD often presents as a Reye's-like syndrome characterized by fasting intolerance with vomiting, recurrent episodes of hypoglycemic coma, hypoketotic dicarboxylic aciduria, low plasma and tissue carnitine, hepatic failure, encephalopathy, and rapid progressive deterioration leading to death. Episodes can be precipitated by any stress which results in decreased caloric intake or increased catabolism, such as intercurrent illness or childhood immunization. If the diagnosis is made early and the patient is carefully managed, the prognosis is generally good.

The MCAD gene was mapped to the short arm of chromosome 1 (1p31) in 1987 (3). In the same year the first indication of the presence of a common mutation was reported by Ikeda *et al.* (4). In 1990, Kelly *et al.* (5) reported a frequently occurring A985G transition mutation resulting in a Lys to Glu substitution at amino acid 329 in the precursor protein subunit. Further studies showed that 80% of patients were homozygous for this mutation (6), and most of the remaining 20% were compound heterozygotes.

Received December 9, 1994; accepted February 10, 1995
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Supported by grants from Health Resources and Services Administration, Maternal and Child Health training grant MCJ 429169 (E.W.N.), and National Institutes of Health grant NS28403 (E.P.H.).

A number of studies have estimated the frequency of MCAD deficiency in various European populations based on the number of A985G carriers detected in the population. These data have led to disease frequency estimates ranging from 1/6400 to 1/18,500 live Caucasian births (7, 8) depending on the population tested. These calculations assume that the high frequency of A985G seen in patients who have clinically presented with metabolic crisis and who were subsequently diagnosed biochemically, reflects the mutation allele frequency of heterozygous carriers.

A goal of this study was to prospectively ascertain a cohort of MCAD patients through mass neonatal screening of blood acylcarnitines using MS/MS and to determine disease frequency and approximate allele frequencies based on this random sample. Indeed, we felt the high early mortality of the disease (approximately 50% of cases can be expected to die during the first clinical episode) also made the study particularly important. Presymptomatic detection of MCAD would permit early initiation of treatment in the neonatal period and avoidance of risk factors that can instigate metabolic crisis. Neonatal screening from filter paper blood specimens is mandated for phenylketonuria and congenital hypothyroidism in the United States. In Pennsylvania screening for maple syrup urine disease and sickle cell disease is also required. We have an ongoing supplemental newborn screening program that screens for a number of inherited metabolic disorders including MCAD and other fatty acid and organic acid disorders based on the detection of acylcarnitine derivatives using MS/MS (9, 10). Here we report our experience with the first 80,371 newborns screened for MCADs between 11/1/92 and 9/30/94.

METHODS

Neonatal screening protocol. Neonatal screening was performed Through our supplemental newborn screening program, which currently tests over 80,000 newborns annually in Pennsylvania and Eastern Ohio. Specimens collected by heel stick, spotted on filter paper, are mailed to the central screening laboratory for testing. The supplemental screening program tests for over 35 metabolic disorders by a variety of techniques. Patients with MCAD deficiency were identified using a static liquid secondary ion monitoring tandem mass spectrometry (LSIM-MS/MS) technique developed by Millington *et al.* (9, 10). Two 3/16-inch blood spots are punched into a conical vial. Deuterated acylcarnitine internal standards are added followed by extraction with 400 μL of methanol. After shaking for 30 min the supernatant is transfered to a flat bottom vial and evaporated to dryness under nitrogen. Butyl esters are synthesized with the addition of 50 μL of butanol-HCl and incubated at 65°C for 15 min. Specimens are dried again under nitrogen, and 30 μL of a 1:1 (v:v) methanol:glycerol solution containing 0.1% wt/vol sodium octyl sulfate are added before analysis. Analysis is carried out using a VG Quatro triple-quadrupole MS/MS (Fisons Instruments, Danvers, MA). The method incorporates a cesium ion source and a manual insertion probe.

DNA analysis. Peripheral blood was collected in purple top tubes or on newborn screening filter paper on all patients and parents when available. DNA was isolated from peripheral blood (11) or eluted directly from filter paper by boiling. The common A985G mutation was detected by restriction fragment digestion of PCR product as described by Yokota *et al.* (12), with the exception that the reverse primer used for the amplification was as follows (5′-CCTCCCAAGCTGCTCTCTGG-3′). Oligonucleotide primer pairs covering each exon from the exon/intron boundries were designed (13) to amplify all the MCAD exons. Primers to amplify exon 5 were as follows. Primer 5 FORWARD: 5′-GGGGGGATCCTATTGTGC-CAGCCAGAACAC-3′ and primer 5 REVERSE: 5′-GGG-GAAGCTCCAACTTCTTCAGGAAGTAAC-3′.

Amplification from genomic DNA was performed in 100-μL reactions with 15 pmol of each primer, 2 U of recombinant *Taq* polymerase (Perkin-Elmer Corp., Norwalk, CT), and the following program for 30 cycles: 94°C for 3 min, 55°C for 2 min, and 72°C for 3 min. Aliquots of the PCR products were denatured and loaded on nondenaturing gels for SSCP analysis (14) using hydrolink MDE gels. Electrophoresis was performed at room temperature at 8 W constant power for 14 h. Gels were dried and autoradiographed for 10–16 h with an intensifying screen at −80°C. Conformers visualized on dried SSCP gels were excised and rehydrated in TEN. DNA was eluted overnight and desalted using Sephadex G25 spin columns. Biotinylated forward primers and unmodified reverse primers were used to reamplify the purified DNA. Single stranded DNA was purified using streptavidin coated magnetic beads (DYNA BEADS). Sequencing reactions were carried out using Sequenase (U.S. Biochemical Corp., Cleveland, OH) and S^{35}-nucleotide precursors. Electrophoresis was performed on 6% denaturing polyacrylamide gels for 6 h, and autoradiography was carried out for 12–48 h.

RESULTS

Nine newborns testing positive for MCAD were detected prospectively using LSIM-MS/MS in the first 80,371 newborns screened as of September 30, 1994, by detecting a characteristic abnormal pattern on the acylcarnitine profile which showed markedly elevated levels of octanoylcarnitine (C8), hexanoylcarnitine (C6), and decanoylcarnitine (C10) (Fig. 1). DNA analysis was completed on all nine of these affected newborns and showed only four of these MCAD probands to be homozygous for the A985G transition (including a pair of siblings; K.B. and D.B.), whereas the other five were compound heterozygotes (Table 1). We also diagnosed two additional newborns with MCAD deficiency as a result of a positive family history including one that was a first cousin (N.P.) of a proband detected by routine screening (A.Z.). These two cases were born at hospitals not part of our supplemental screening program and therefore are not included in our frequency calculations, but are included as part of our molecular studies. Screening with SSCP for the other mutations showed a conformer on exon 5 of the MCAD gene in one of the affected newborns (AM) (Fig. 2). Her father was a carrier of the A985G mutation, whereas her mother carried the same exon 5 conformer on SSCP analysis. Sequencing the conformer revealed a 6-bp deletion that involves two amino acids, glycine and cysteine (Fig. 3). A second rare mutation was identified on

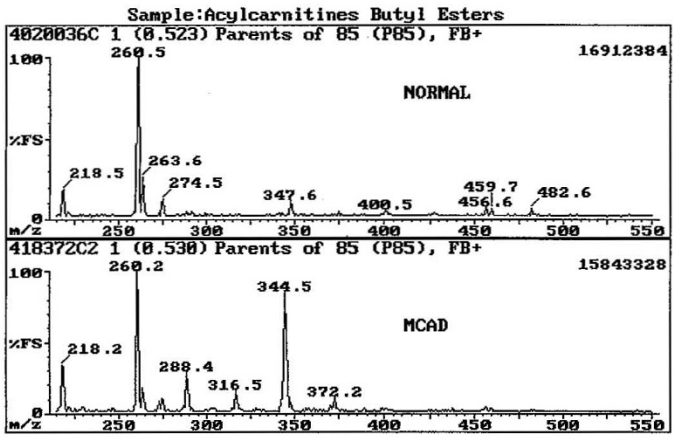

Figure 1. Neonatal screening of blood spots for MCAD by LSIM-MS/MS acylcarnitine profiles. Shown is a profile from a normal neonate (top panel), with primarily acetylcarnitine (m/z 260). Deuterated internal standards (m/z 263, 247, 459) are also seen. The profile from a neonate with MCAD shows abnormal octanoyl carnitine (m/z 344), butyrylcarnitine (m/z 288), hexanoylcarnitine (M/Z 316), and decanoylcarnitine (m/z 372). All nine patients detected through neonatal screening showed similar LSIM-MS/MS acylcarnitine profiles.

Table 1. *DNA analysis*

MCAD patients	Date of birth	DNA analysis
Prospective		
K.B.	12–8 -92	985/985
A.Z.	12–17-92	985/other
K.E.	1–10-93	985/other
L.H.	4–18-93	985/other
A.M.	5–20-93	985/6bp deletion
S.L.	10–10-93	985/exon7
J.S.	3–25-94	985/985
N.D.	5–2 -94	985/985
D.B.	6–10-94	985/985
Family history		
C.M.	9–23-92	985/583
N.P.	12–28-92	985/other

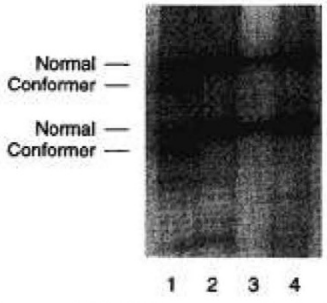

Figure 2. Detection of a potential MCAD gene mutation by SSCP analysis in exon 5. Shown is SSCP analysis of exon-specific PCR products of genomic DNA from MCAD patients (lanes 1, 2, and 3) and a normal control (lane 4). One MCAD patient shows a unique conformer, which was excised from the gel and sequenced.

exon 7 and this is currently being sequenced. Among the other cases detected, a compound heterozygote was identified with the A985G and a G583A allele (Strauss).

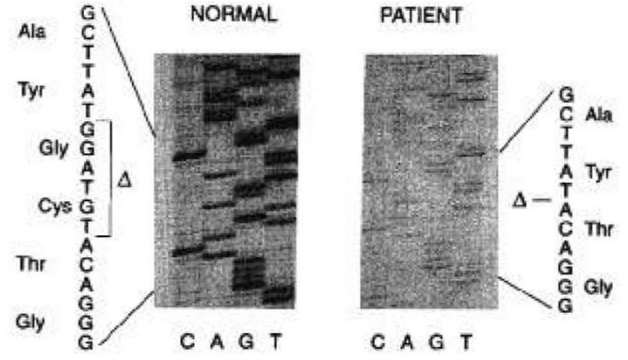

Figure 3. DNA analysis showing a novel 6-bp (two amino acids) deletion mutation. Shown is sequence analysis of the PCR product corresponding to the SSCP conformer seen in exon 5 (patient; right panel) and a normal control (left panel). A 6-bp deletion is seen in the sequence of the patient.

DISCUSSION

We have established a neonatal screening protocol for MCAD using MS/MS detection of acylcarnitine derivatives from blood spots. Our initial screen of 80,371 newborns detected nine MCAD patients with the expected marked elevations of octanoylcarnitine, hexanoylcarnitine, and decanoylcarnitine. Our results are significant at a number of levels. First, we report the first determinations of incidence of MCAD based on prospective biochemical analysis of random neonatal blood spots, and we found this incidence to be 1/8,930. Previous estimates of the incidence of MCAD have been based on population screening of asymptomatic carriers for the A985G mutation, which has previously been reported to be responsible for 90% of MCAD mutation alleles in retrospectively identified patients (7, 8). The A985G mutation is thought to have originated in the Western European population, and is prevalent in German, English, and Scottish peoples. The population we screened is heterogeneous, including high proportions of Eastern Europeans and Blacks. Despite this diverse population, we still found MCAD to be one of the most common inherited metabolic disorders of humans.

Second, we found the allele distribution in our prospective random screening to be substantially different from those published previously in retrospective clinical studies: others found 81% of patients to be homozygous for the A985G mutation, and 18% to be heterozygous; we found 44% (4/9) of our patients to be homozygous and 56% (5/9) to be heterozygous. There are many possible explanations for this allele frequency difference. It is possible that only a subset of MCAD patients are identified clinically; many patients die suddenly in early childhood, and many may go undiagnosed after death. The allele frequency differences suggest that many MCAD patients may show either very severe, or very mild symptoms: our neonatal screening protocol should detect all biochemically deficient patients, whereas retrospective clinical ascertainment may detect only those showing classical symptoms. If true, then A985G homozygotes may show a clinical phenotype which is distinct from most compound heterozygotes. Alternatively, there may be a second mutant allele that has become established within the Pennsylvania area. We feel this is unlikely given the diverse ethnic background of the region.

Furthermore, we have identified three of the six non-A985G mutant alleles, and none of these is more frequent in our A985G heterozygotes. Other possibilities are less likely: asymptomatic heterozygotes for MCAD show normal MS/MS profiles, and it is therefore unlikely that we have detected carriers in our screen.

Third, we identified a novel mutation during the course of this study, a 6-bp deletion which removes two amino acids from the enzyme (delG90/C91). These two amino acids are located in the β loop of helix D of the enzyme and are far from the active site; however, it is likely that the deletion results in an unstable protein susceptable to degradation (15). We screened four of our five A985G heterozygote patients by exon-specific SSCP to detect the other mutant allele. Approximately 90% of the coding sequence was screened, and we detected two conformers: one in exon 5 that we found to be the 6-base deletion, and one in exon 7 that we have not yet characterized. It is important for future studies to characterize the remaining mutations in these heterozygote patients, determine the enzyme activities of novel mutations, and test the hypothesis that MCAD patients heterozygous for A985G can show unexpectedly severe or mild phenotypes.

Finally, neonatal screening for MCAD permits presymptomatic ascertainment of patients, with subsequent parent and physician counseling designed to teach the prevention and recognition of metabolic crises. Patients are given a low fat, high carbohydrate diet to minimize Reye's-like episodes, and parents are provided with genetic counseling and prenatal diagnosis if desired. Despite early identification, two infants we identified died suddenly. One child, homozygous for the G985A mutation, entered a metabolic crisis after an immunization. The second, a compound heterozygote, died during an intercurrent illness. With increasing awareness of this condition in the medical community and continued emphasis on parent education we hope that mortality rate in this condition will decline.

Acknowldgements. The authors thank Dr. Jung-Ja P. Kim, Dr. Holmes Morton, Dr. Dave Millington, and Dr. Don Chace for discussion and advice, and Brenda Mitchell, R.N., for her assistance in tracking patients and newborn specimens.

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