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Abstract We report a novel mild variant of medium-chain acyl-CoA dehydrogenase deficiency (MCADD) diagnosed in four infants who, in neonatal screening, showed abnormal acylcarnitine profiles indicative of MCADD. Three patients showed completely normal urinary organic acids and phenylpropionic acid loading tests were normal in all four patients. Enzyme studies showed residual MCAD activities between “classical” MCADD and heterozygotes. *ACADM* gene analysis revealed compound heterozygosity for the common mutation K329E and a novel mutation, Y67H, in two cases, and homozygosity for mutation G267R and the novel mutation S245L, respectively, in two children of consanguineous parents. As in other metabolic disorders, the distinction between “normal” and “disease” in MCAD deficiency is blurring into a spectrum of enzyme deficiency states caused by different mutations in the *ACADM* gene potentially influenced by factors affecting intracellular protein processing.

Introduction

The mitochondrial beta-oxidation of activated fatty acids (acyl-CoA esters) is essential for the provision of energy to the cell, especially during fasting periods. Genetic beta-oxidation defects typically present in early childhood with potentially life-threatening hypoketotic, hypoglycaemic coma

during catabolic states, e.g. prolonged fasting or infections. The most frequent defect in this group affects the enzyme medium-chain acyl-CoA dehydrogenase (MCAD, EC 3.1.2.20), which is required for the oxidation of medium-chain (C6–C10) acyl-CoA esters. MCAD deficiency (MCADD, OMIM 201450) is caused by mutations in the *ACADM* gene and is inherited as an autosomal recessive trait. Among symptomatic infants with the disorder, up to one quarter do not survive the first acute metabolic decompensation; others may stay non-symptomatic throughout life (Pollitt and Leonard 1998). The exact prevalence of MCADD in Europe is not known but may exceed 1:10,000 in some regions (Seddon et al. 1995; Tanaka et al. 1997). One prevalent mutation in the *ACADM* gene, K329E, accounts for up to 90% of mutant chromosomes identified so far. Carrier studies for K329E in the general population have indicated that a significant proportion of individuals with MCADD in many countries remain undiagnosed either because the diagnosis is missed or because they remain non-symptomatic (Tanaka et al. 1997). This is corroborated by the emerging results of neonatal screening programs for MCADD by using tandem mass spectrometry (tandem-MS); these programs have provided prevalence figures that are even higher than estimates based on mutation frequencies (Chace et al. 1997).

The diagnosis of MCADD traditionally involves the analysis of urinary organic acids; this may show the excretion of hexanoylglycine, suberylglycine and phenylpropionylglycine in addition to various dicarboxylic acids. However, urinary organic acids may be normal, particularly in non-fasting states. Acylcarnitine analysis in dried blood spots has recently been introduced as a more sensitive test that can also be used for neonatal mass screening (Chace et al. 1997). The diagnosis may be confirmed by enzyme or mutation studies but frequently a phenylpropionic acid (PPA) loading test is carried out as a rapid and specific *in vivo* test (Rumsby et al. 1986). PPA is a non-toxic substance that is also produced by normal gut bacteria and requires MCAD for its oxidation to hippuric acid. Patients with MCADD fail to metabolise PPA and excrete large amounts of phenylpropionylglycine after PPA load-

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ing. The test is regarded as safe and reliable for the diagnosis of MCADD (Glasgow et al. 1992; Lehnert 1993).

Since the start of neonatal screening for organic acidurias and fatty acid oxidation defects, we have identified several infants with abnormal acylcarnitine profiles suggestive of MCADD but with normal urinary organic acids and normal results in PPA loading tests. We now report that some of these patients suffer from a mild form of MCADD caused by compound heterozygosity for the common mutation K329E and another, presumably mild mutation, or by homozygosity for such a mutation. A mild enzymatic phenotype has been confirmed by in vitro studies showing significant residual MCAD activity in the cells of the patients.

Patients and methods

Patients

Patients included four children who, in routine neonatal screening on the 5th day of life, were found to have an abnormal acylcarnitine profile indicative of MCADD (see below). Patients 1 and 2 were of German origin, whereas patients 3 and 4 were born to different consanguineous Turkish parents. The clinical course and routine laboratory investigations up to the age of 6 months were unremarkable in all subjects. All investigations were performed with informed consent.

Acylcarnitines

Acylcarnitines in dried blood spots obtained for neonatal screening were analysed on a triple quadrupole tandem mass spectrometer with an ion spray source (API 365, PE Sciex, Canada) as previously described (Rashed et al. 1995; Schulze et al. 1999). The individual compounds were detected by searching for the precursor ions of $m/z=85$, quantified by using the signal intensity ratio to the closest internal standard, and related to concentrations by using the slope derived from standard curves.

Urinary organic acids

Urinary organic acids were analysed by gas chromatography-mass spectrometry (GC-MS) as previously described (Hoffmann et al.

1989). The detection limit for hexanoylglycine, the most characteristic indicator of MCADD, was less than 1 mmol/mol creatinine, below the upper normal limit of 1.2 mmol/mol creatinine.

PPA loading test

For the PPA loading test, all patients received 25 mg/kg body weight PPA mixed in tea (Rumsby et al. 1986). Urine was collected before the test and for a period of 6–8 h after the test and analysed.

MCAD enzyme activity

MCAD enzyme activity was measured in lymphocyte homogenates and cultured fibroblasts by using the specific substrate phenylpropionyl-CoA and quantitation of the reaction product by high pressure liquid chromatography as previously described (Wanders et al. 1999).

Molecular genetic studies

All exons and adjacent intron segments of the *ACADM* gene were polymerase chain reaction (PCR)-amplified from genomic DNA with previously described primers (Andresen et al. 1997) and sequenced on a fluorescent sequencer (Alf Express, Pharmacia). Mutation Y67H was analysed by *Nla*III restriction enzyme digests of the normal PCR product containing exons 3 and 4; the mutation creates a restriction site that cleaves the 424-bp product into two fragments of 157 bp and 267 bp. Mutation S245L was examined by single-strand conformation polymorphism analysis of PCR products of exons 9 by using a silver-staining method (Multiphor II, Pharmacia) in which it produced a clearly visible band shift.

Results

Neonatal acylcarnitine profiles were highly abnormal in all four patients and were suggestive of MCADD (Table 1). Specifically, the concentration of octanoylcarnitine was increased, whereas there was no accumulation of carnitine species greater than 10:1 (decanoyl) or less than C6 (hexanoyl). The ratios C8/C12 (octanoyl/dodecanoyl), C8/C10 (octanoyl/decanoyl) and C8/C2 (octanoyl/acetyl) were all clearly elevated. Even if, in neonates, the concentration of

Table 1 Neonatal acylcarnitine concentrations and relative molar ratios in blood spots from screening cards of a healthy control population, patients with classical MCADD (e.g. homozygous for K329E), and patients with mild MCADD (all quantitative values are given in $\mu\text{mol/l}$)

	Acylcarnitines/ratios							
	Acetyl (C2)	Hexanoyl (C6)	Octanoyl (C8)	Decenoyl (C10:1)	Decanoyl (C10)	C8/C2	C8/C10	C8/C12
Controls ($n=2650$)								
Median	12.95	0.26	0.14	0.12	0.14	0.010	1.00	0.71
95th Percentile	21.11	0.40	0.28	0.25	0.27	0.021	2.90	1.85
Classical MCADD ($n=6$)								
Minimum	7.84	0.20	1.03	0.08	0.05	0.080	2.87	3.13
Mean	13.57	0.74	3.38	0.42	0.52	0.213	7.45	15.57
Maximum	18.16	1.61	7.14	1.00	1.46	0.470	19.55	41.00
Mild MCADD								
Patient 1	12.08	0.48	1.85	0.38	0.73	0.150	2.58	7.69
Patient 2	10.75	0.71	1.55	0.33	0.42	0.143	3.72	8.89
Patient 3	7.25	0.77	1.16	0.51	0.16	0.160	7.25	8.45
Patient 4 ^a	7.50	0.72	2.71	1.36	0.52	0.345	5.29	21.90

^aAcylcarnitine data from patient 4 were obtained at age 4 months

Table 2 Activity of medium-chain acyl-CoA dehydrogenase in lymphocytes (patients 1, 3 and 4) and fibroblasts (patient 2) of our four patients with mild MCADD, as well as in patients with classical MCADD, heterozygous carriers for MCAD mutations, and controls

	Activity (mol/min·mg)	Percentage of controls	Genotype
Mild MCADD			
Patient 1 (lymphocytes)	0.17	17%	K329E/Y67H
Patient 2 (fibroblasts)	0.5	10%	K329E/Y67H
Patient 3 (lymphocytes)	0.10	10%	S245L homozygous
Patient 4 (lymphocytes)	0.09	9%	G267R homozygous
Classical MCADD			
Fibroblasts (<i>n</i> =8)	0.001±0.001	<0.5%	K329E homozygous
Lymphocytes (<i>n</i> =2)	0.03; 0.05	<5%	K329E homozygous
Heterozygous carriers			
Fibroblasts (<i>n</i> =2)	0.21; 0.22	40–45%	K329E heterozygous
Lymphocytes (<i>n</i> =3)	0.44±0.10	35–50%	K329E heterozygous
Lymphocytes (<i>n</i> =2)	0.47; 0.52	45–50%	G267R heterozygous
Lymphocytes (<i>n</i> =7)	0.49±0.15	35–60%	Other mutations
Controls			
Fibroblasts (<i>n</i> =16)	0.50±0.16	–	Wild-type
Lymphocytes (<i>n</i> =26)	1.02±0.41	–	Wild-type

octanoylcarnitine and the ratio of C8/C12 in patients with mild MCADD tend to be lower than in patients with classical MCADD, one cannot discriminate these two forms on the basis of the neonatal acylcarnitine profile in individual cases. Acylcarnitine data obtained after the neonatal period remained abnormal in all subjects with mild MCADD, however, the abnormalities were not as marked as in patients with classical MCADD (data not shown).

Urinary organic acid analyses yielded normal results (in particular, no detectable hexanoylglycine) in patients 1, 2 and 4 but showed elevations of hexanoylglycine and several dicarboxylic acids in patient 3. The specific metabolites 5-hydroxyhexanoic acid or suberylglycine were not identified in any of the patients. After PPA loading, all patients showed excretion of large amounts of hippuric acid but no excretion of phenylpropionylglycine, reflecting adequate ingestion and suggesting normal beta-oxidation of PPA and sufficient MCAD function.

MCAD enzyme analyses showed marked enzyme deficiency in all patients (Table 2). However, the values obtained were unusual as they were higher than those in other patients with MCADD but much lower than those in heterozygous carriers. The reliability of these findings, i.e. truly elevated residual MCAD activity, was confirmed through titration experiments combining aliquots of lysates prepared from classical MCADD patients and our patients (data not shown).

Molecular genetic studies in patients 1 and 2 revealed compound heterozygosity for the common mutation K329E (c.985A→G) and a novel mutation, Y67H in exon 3 of the *ACADM* gene. This substitution of histidine for tyrosine at amino acid residue 67 is caused by a T→C alteration at nucleotide c.199. The mutation was not found in 120 normal control chromosomes. Patient 3 was homozygous for a C→T alteration at nucleotide c.734 in exon 9, causing a substitution of leucine for serine at residue 245 (mutation

S245L). The mutation was not found in 120 normal control chromosomes. Patient 4 was homozygous for the previously described mutation G267R (c.799G→A). No other mutation was identified in the whole coding region of the *ACADM* gene in either child. Inheritance of the mutations in *trans* was confirmed in each family through molecular analyses of both parents.

Discussion

We report a novel mild variant of MCADD associated with unusual biochemical findings, significant residual enzyme activity and novel mutations in the *ACADM* gene. Serine at residue 67 is located at the periphery of the MCAD tetramer and is not conserved compared with other acyl-CoA dehydrogenases. In contrast, both glycine²⁶⁷ and serine²⁴⁵ are highly conserved in humans and other organisms and are found at the equivalent positions in human short-chain and branched-chain acyl-CoA dehydrogenases. Mutation G267R (in previous reports denoted G242R) has been previously reported in association with K329E in patients with symptomatic MCADD (Yokota et al. 1991; Andresen et al. 1997). It affects a glycine residue conserved in several other human acyl-CoA dehydrogenases. Expression studies of G267R in *Escherichia coli* (Andresen et al. 1997) have revealed considerable residual MCAD activity of the G267R protein. The values measured were similar to those of the K329E protein but the thermal stability of the K329E protein was decreased compared with the wild-type, whereas the thermal stability of the G267R protein was normal. It is possible that the relatively high residual MCAD activity in our patient homozygous for G267R is attributable to factors affecting cellular protein processing. In vitro MCAD activities of both G267R and K329E proteins were markedly increased by as much as 40% of normal when co-expressed with chaperonins GroEL and

GroES, indicating that both mutations affect protein folding (Andresen et al. 1997).

The most sensitive diagnostic test for mild MCADD is acylcarnitine analysis by tandem-MS, which provided consistently abnormal results in our patients. Organic acid analysis may be normal or may show mild dicarboxylic aciduria; the diagnostic metabolites 5-hydroxyhexanoic acid and suberylglycine were not detected in our patients. Of great clinical relevance are the false negative results of the PPA loading tests, previously thought to be highly sensitive for the detection of MCADD. Beta-oxidation capacity for medium chain acyl-CoA compounds (including phenylpropionyl-CoA) in our patients appears to be sufficient for the full conversion of small to moderate amounts of substrate, prohibiting the accumulation of abnormal metabolites after administration of a standard PPA dose. The test gives only approximately 165 μmol (25 mg) PPA per kg body weight, which is relatively low compared with conditions during fasting when free fatty acids may reach steady-state concentrations of several millimoles per litre in plasma. Similarly, large amounts of fatty acids are included in the normal diet. During a standardised oil challenge, 1.5 g sunflower oil per kg body weight is given, which is equivalent to a dose of more than 5 mmol/kg body weight, with respect to the molecular weight of oleic acid. It is likely that pathological metabolites would be observed in individuals with mild MCADD if much greater amounts of PPA were administered.

The clinical relevance of mild MCADD is as yet unknown but difficult to establish. It is uncertain whether mild MCADD as observed in our patients carries a risk for significant or life-threatening metabolic decompensations. None of our patients up to the age of 6 months has experienced hypoglycaemia or any other symptom that may be attributed to impaired fatty acid oxidation. However, this observation period is very short and all parents were advised to avoid prolonged fasting periods. Three of the four patients were prescribed carnitine, although there was no evidence of carnitine deficiency in these patients (data not shown). Two of our patients were compound heterozygous for the same novel mutation Y67H in addition to the common mutation K329E; it is possible that other patients with this genotype remained undetected because they had not yet experienced any adverse effects of mild MCAD enzyme deficiency.

Nevertheless, in view of the physiological requirement to oxidise large amounts of fatty acids during prolonged fasting periods and considering that some patients, even with classical severe MCADD, stay asymptomatic throughout life, it appears possible that individuals with mild MCADD may run into severe problems of energy and glucose homeostasis or carnitine depletion under certain circumstances. It is important to note that mutation G267R has been previously identified in symptomatic patients (Yokata et al. 1991; Andresen et al. 1997). G267R, therefore, cannot be regarded as a trivial genetic variant but may be of pathogenetic relevance, at least in association with the K329E mutation.

In conclusion, in MCAD deficiency as in many other metabolic disorders, the distinction between "normal" and "disease" is becoming blurred into a spectrum of enzyme deficiency states caused by different mutations in the *ACADM* gene and being potentially influenced by factors affecting intracellular protein processing. The increasing use of acylcarnitine analysis in neonatal screening will probably result in the identification of more subjects with mild MCADD caused by homozygosity or compound heterozygosity for mutations with residual enzyme function. Further functional investigations of such mutations and the genetic characterisation of as many clinically presenting MCADD patients as possible should provide reliable information on the potential risks associated with this condition.

Electronic-database information

- The Genome Database (GDB): 118958, <http://gdbwww.gdb.org/gdb-bin/genera/accno?GDB:118958>
- Human Gene Mutation database (HGMD): *ACADM*, <http://archive.uwcm.ac.uk/uwcm/mg/search/118958.html>
- Online Mendelian Inheritance in Man (OMIM): *ACADM*, <http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=201450>

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