SHORT REPORT

Combined malonic and methylmalonic aciduria: exome sequencing reveals mutations in the ACSF3 gene in patients with a non-classic phenotype

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ABSTRACT

Background Combined Malonic and Methylmalonic Aciduria (CMAAMMA) is a rare recessive inborn error of metabolism characterised by elevations of urine malonic acid (MA) and methylmalonic acid (MMA). Nearly all reported cases are caused by malonyl-CoA decarboxylase (MCD) deficiency. Most patients have metabolic acidosis, developmental delay, seizures and cardiomyopathy. CMAMMA was also described in symptomatic patients with normal MCD activity, suggesting heterogeneity in this disorder.

Methods and results We identified two probands with a non-classical CMAMMA variant through the Quebec newborn urine screening program. While they share the biochemical phenotype of elevated MA and MMA, the MMA excretion was higher than MA, the clinical courses were benign, MYLCD gene sequencing was normal and MCD activity, measured in one proband, was normal. Using exome sequencing in the single consanguineous proband, we identified a homozygous missense allele in the ACSF3 gene, encoding an Acyl-CoA Synthetase (ACS) with unknown substrate and function. The second proband was homozygous for a different ACSF3 missense allele. Both substitutions were in conserved residues and were identified in less than 0.5% of their respective ethnic control populations.

Conclusion These results suggest that ACSF3 is a candidate gene for non-classical CMAMMA observed in our patients and document the value of exome sequencing of a limited number of patients for the identification of novel disease genes.

Several types of patients with elevated malonic acid (MA) have been described in the literature. The most well characterised is that related to malonyl-coenzyme A (CoA) decarboxylase (MCD) enzyme deficiency (OMIM #248360) in which the biochemical abnormalities include elevated MA alone, or combined elevations of MA and methylmalonic acid (CMAMMA) with MA mainly being higher than methylmalonic acid (MMA).1-11 It is speculated that MCD deficiency causes an excess of intramitochondrial malonyl-CoA, leading to the inhibition of methylmalonyl-CoA mutase and, subsequently, an increase in MMA. Two patients have been described with similar combined elevations of MA greater than MMA, but with normal MCD enzyme activity in fibroblast culture.12, 13 suggesting that this disorder is heterogeneous. All of these patients have shown a variety of clinical features that include metabolic acidosis, developmental delay, seizures, cardiomyopathy, gastrointestinal distress and dysmorphic features, although normal development was also reported.11 Through the Quebec newborn urine screening programme we identified asymptomatic individuals with CMAMMA, but with MMA higher than MA, and sought to define the underlying molecular cause. We refer to these patients as having non-classic CMAMMA.

METHODS

Metabolite detection

The Quebec newborn urine screening programme, including MMA since 1975, evaluates urine from 4–6-week-old infants by thin layer chromatography. Subsequent urine organic acid analysis by gas chromatography mass spectroscopy confirmed CMAMMA. MMA is considered elevated when there is more than 200 μmol/mmol creatinine in the newborn period.15 Patients were followed in our medical genetics clinic and informed consent for research studies was obtained according to institutional guidelines.

Molecular analysis

Genomic DNA was isolated from patient fibroblast cell lines. All exons and intronic flanking sequences of MLYCD were amplified and sequenced by Sanger methods; primers and PCR conditions were modified from Wightman et al.11 ACSF3 exons 5 and 8 were amplified from gDNA in 31 controls using primers and conditions reported here (see supplementary material, available online only). GenBank ref seq nos: MLYCD, NM_012213; ACSF3, NM_174917; TAS2R43, NM_176884.

Exome sequencing

A total of 3 μg of DNA was subjected to the SureSelect Human All Exon v2 Kit from Agilent Technologies (Santa Clara, CA, USA), according to the manufacturer’s protocols. This kit targets approximately 44 Mb of sequence including the human exome defined by the NCBI consensus coding sequence database (version 20090902) and additional ref seq sequences. The captured DNA was sequenced by Illumina GAIIx (San Diego, CA, USA) on the one lane, generating approximately 32 million
RESULTS

The clinical and biochemical features of the two non-classic CMAMMA probands in this report are described below.

Patient 1, a 14-year-old boy, was born to non-consanguineous Ashkenazi Jewish parents (figure 1A, II:3) and was briefly reported previously.15 His initial urine MMA level was 891 μmol/mmol creatinine. Confirmatory testing by gas chromatography mass spectroscopy showed a urine MMA of 745 μmol/mmol creatinine (normal <25) and MA of 60 μmol/mmol creatinine (normal <6). His plasma MMA level was 18 μmol/l (normal <0.5). On follow-up the ranges of urine MA and MMA were 20–68 and 214–470 μmol/mmol creatinine, respectively; the plasma MMA range was 20–32 μM.

Patient 2, a 4-year-old girl, was born to first cousin French Canadian parents (figure 1B, II:1). Her initial MMA level was 800 μmol/mmol creatinine, and confirmatory testing showed urine MMA and MA levels of 757 and 67 μmol/mmol creatinine, respectively; the plasma MMA level was 59.2 μmol/l. On follow-up the ranges of urine MA and MMA were 43–260 and 690–1830 μmol/mmol creatinine, respectively. The range of plasma MMA was 28–40 μM. She has a 2-year-old similarly affected sibling (figure 1B, II:2) with urine MMA 843 and MA 94 μmol/mmol creatinine.

Both patients were born at term, were clinically asymptomatic, had normal cardiac examinations and age-appropriate development. No other organic acid abnormality was detected. Carnitine levels, acylcarnitine profiles, plasma amino acids, ammonia, blood gas values, total homocysteine, folate and vitamin B12 levels were all within reference ranges, and the results were within the reference range.

Molecular analysis

No mutations in MYLCD were found in either patient. Exome sequencing of patient 2, followed by the selection of novel non-synonymous variants (see Methods) that were either homozygous or potentially compound heterozygous (two mutations in the same gene) resulted in the identification of only two potential candidate genes: TAS2R43 (taste receptor, type 2, member 43) and ACSF3, a novel member of the family of acyl-CoA synthetases (ACS). TAS2R43 encodes a seven transmembrane G-protein-coupled receptor located on the surface of taste receptor cells and mediates cell responses to bitter taste.20 A homozygous missense substitution in a residue conserved among mammals, c.97A→G (p.Ile33Val), was identified and predicted to be located in a potential cytoplasmic domain. We did not detect any region of homozygosity around this variant. However, based on potential biological significance, ACSF3 was selected as the more likely candidate. Patient 2 was homozygous for c.1075G→A in exon 5, predicted to substitute a glutamate to a lysine residue, or p.Glu559Lys. The glutamate residue is highly conserved through evolution among all ACSF3 homologues, including zebrasfish, neurospora and rice, and this substitution was predicted to be damaging based on PolyPhen-2 functional analysis.21 This allele was annotated in 1000 Genomes with a rare allele frequency of 0.001. There were no heterozygous SNP in the vicinity of this mutation, suggesting a region of homozygosity spanning approximately 5.4 Mb at the tail of chromosome 16q. This mutation was also identified in homozygosity in the affected brother. In order to determine whether ACSF3 play a role in the patients’ phenotype, ACSF3 was sequenced in patient 1, and a homozygous mutation, c.1411C→T (p.Arg471Trp), was identified in exon 8. The arginine residue is again highly conserved in evolution and the substitution was predicted to be damaging.21 The Sanger

Exomes

Figure 1 Pedigrees of (A) family 1 and (B) family 2. Blackened boxes indicate the presence of non-classic combined malonic and methylmalonic aciduria and homozygosity for ACSF3 mutations. Parents were heterozygote carriers of the identified mutations. Parents and unaffected siblings had normal urine malonic acid and methylmalonic acid levels; unaffected siblings were not genotyped.
sequence traces for both mutations and parental genotypes are shown in figure 2.

To determine the frequency of the missense alleles in an ethnically matched population, we sequenced ACSF3 exon 5 in 139 French Canadian controls and exon 8 in 176 Ashkenazi Jewish controls. The c.1075G→A (exon 5) mutation was found in a heterozygous state in one French Canadian control individual, predicting a minimum incidence of this phenotype to be one in 77,284 in this population. The c.1411C→T (exon 8) mutation was found in a heterozygous state in a single Ashkenazi Jewish control individual, predicting a minimum incidence of this phenotype to be one in 125,094.

**DISCUSSION**

CMAMMA is a rare recessive phenotype characterised by elevated MA and MMA in body fluids. The non-classic patients described here differ from classic patients by having a benign clinical course. When the urine MA levels are compared they overlap; the range of MA are approximately 15–260 in non-classic and 19–600 μmol/mmol creatinine in classic patients. However, the MMA levels in non-classic patients range from approximately 400 to 1000 μmol/mmol creatinine, which is higher than the classic range of 5–300 μmol/mmol. The detection of these patients may be unique to the Quebec newborn urine screening programme. Most newborn screening programmes analyse dried blood spots using tandem mass spectrometry in the first week of life. Infants with classic CMAMMA are suspected by elevations of C3-DC (malonyl) acylcarnitine. It is not known whether non-classic CMAMMA are suspected by elevations of C3-DC (malonyl) acylcarnitine. Previous studies have suggested the activation of fatty acids by forming a thioester with CoA and a fatty acid, to form acyl-CoA. Recently, 26 distinct ACS were identified in the human genome based on the presence of five conserved amino acid motifs. ACSF3 could not be assigned to any of the established subfamilies (ACSS, ACSM, ACSL, ACSVL, or ACSBG) and probably represented a novel ACS family member. Watkins *et al.* demonstrated that ACSF3 did indeed have ACS activity in a heterologous expression system. p.Arg471 located in ACS motif II and mutated in patient 1, represents an ATP binding site by homology and is nearly invariant in all ACS families. Substitution of the equivalent residue in *Escherichia coli* fatty ACS to an alanine (fadD, p.Arg453Ala) resulted in the complete loss of enzymatic activity.

We suggest that defects in ACSF3 are good candidates for the biochemical phenotype in these patients. Future studies will need to be performed to determine whether ACSF3 deficiency is causative of the non-classic CMAMMA described here. Given a putative role as a mitochondrial ACS enzyme, we speculate that ACSF3 might encode a malonyl or methylmalony-CoA synthetase. Previous studies have suggested the possibility of a methylmalony-CoA synthetase providing a ‘free shunt’ for MMA in the mitochondria. For malonyl-CoA synthetase, it is interesting to note that the ACSF5 protein has 55% identity (amino acids 48–573) with bacterial malonyl-CoA synthetases using position-specific iterated BLASTp algorithms.

Exome sequencing is a method to identify novel disease genes using one or a few affected individuals. In this case, the

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**Figure 2** Sanger sequence traces in the mutation region for patients 1, 2, parents and control.

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finding of a homozygous missense allele in ACSF3, facilitated by consanguinity, was corroborated by finding a different homozygous missense allele in the same gene in a second proband. This highlights the growing power of this technique to find novel candidate disease genes with limited numbers of patients and families.

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**Competing interests** None.

**Contributors** AA, NB, KAT, JM and SM identified and characterised the patients; LDN, NA and DSR performed the biochemical and molecular studies; AA and NB wrote the paper; LDN, KHH and JM performed the exome sequencing and analysis; LDN, NA and DSR. Contributors AA, NB, KAT, JM and SM identified and characterised the patients; LDN, NA and DSR performed the biochemical and molecular studies; AA and NB wrote the paper; LDN, DSR, KHH and JM contributed to the final manuscript; NB is the guarantor.

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