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. CMAAMMA 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 CMAAMMA variant through the Quebec newborn urine screening program. While they share the biochemical phenotype of elevated MA and MMA, the MCD excretion was higher than MA, the clinical courses were benign, and gene sequencing was normal and MCD activity 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 CMAAMMA 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 (CMAAMMA) with MA being higher than methylmalonic acid (MMA). 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, 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. Through the Quebec newborn urine screening programme, we identified asymptomatic individuals with CMAAMMA, but with MMA higher than MA, and sought to define the underlying molecular cause. We refer to these patients as having non-classic CMAAMMA.

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 confirms CMAAMMA. MMA is considered elevated when there is more than 200 μmol/mmol creatinine in the newborn period. 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. ACSF3 exons 5 and 8 were amplified from gDNA in 515 controls using primers and conditions reported here (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 one lane, generating approximately 32 million reads.
76 bp quality-controlled reads. Reads were aligned to the human reference genome (University of California, Santa Cruz (UCSC) hg19 assembly) using Burrows-Wheeler Aligner’s Smith-Waterman Alignment (BWA). Reads that mapped non-uniquely or to unfinished chromosomes were discarded from further analysis.

Approximately 28.9 million reads were uniquely mapped to the reference genome, providing an average NCBI consensus coding sequence coverage of 25X. We imposed similar variant identification filtering criteria as described. Variants were identified using SAMtools. In addition, we compared our variants against a pool of 34 exomes previously sequenced by us, of which three were reported while the remainder are unpublished. Variants seen in two or more individuals were discarded. Functional annotation of the predicted variants was carried out using ANNOVAR, which cross-references variants discarded. Functional annotation of the predicted variants was performed using PolyPhen-2, SIFT, and dbSNP131 and the 1000 Genomes project.

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. 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 mothers were not vitamin B12 deficient. MMA levels were not responsive to protein restriction or vitamin B12 supplementation and therefore these treatments were not instituted. In cultured fibroblasts, the incorporation of [14C]-propionate, [14C]-methylylterehydrolate and [57C]-cyanocobalamin, and the synthesis of both adenosylcobalamin and methylcobalamin were within the reference range. These results excluded a defect in intracellular vitamin B12 metabolism and methylmalonyl CoA deficiency. MCD activity in fibroblasts was measured in patient 1 and the results were within the reference range.

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 homozgyosity 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.

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. A homozygous missense substitution in a residue conserved among mammals, c.97A→G (p.Ile32Val), was identified and predicted to be located in a potential cytoplasmic domain. We did not detect any region of homozgyosity 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 zebrafish, neurospora and rice, and this substitution was predicted to be damaging based on PolyPhen-2 functional analysis. 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 homozgyosity 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. The Sanger
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 77284 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 125094.

**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 ranges 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 5–500 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 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 48–575) with bacterial malonyl-CoA synthetases using position-specific iterated BLASTp algorithms.

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 ACSF3 protein has 55% identity (amino acids 48–575) 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
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.

Ethics approval Ethics approval was obtained from Montreal Children’s Hospital and MUHC.

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

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REFERENCES

### ACSF3 intronic primers and PCR conditions for gene amplicons

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