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Brief communication

# Novel mutations in a Thai patient with methylmalonic acidemia

Voraratt Champattanachai,[a,1] James R. Ketudat Cairns,[a,b,1] Vorasuk Shotelersuk,[c] Siriporn Keeratichamroen,[a] Phannee Sawangareetrakul,[a] Chantragan Srisomsap,[a] Verachai Kaewpaluek,[d] and Jisnuson Svasti[a,e,*]

[a] *Laboratory of Biochemistry, Chulabhorn Research Institute, Vipavadee-Rangsit Highway, Bangkok 10210, Thailand*
[b] *Schools of Chemistry and Biochemistry, Institute of Science, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand*
[c] *Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand*
[d] *Department of Forensic Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand*
[e] *Department of Biochemistry and Center for Protein Structure and Function, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, Thailand*

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**Abstract**

A Thai patient with methylmalonic acidemia (MMA) and no methylmalonyl-CoA mutase (MCM, EC 5.4.99.2) activity in leukocytes in the presence of deoxyadenosyl cobalamin (*mut*$^0$) was found to be heterozygous for two novel mutations: 1048delT and 1706_1707delGGinsTA (G544X), inherited from her mother and father, respectively. The proband was also heterozygous for the polymorphism, A499T, which did not affect the activity of recombinant MCM.
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## Introduction

Methylmalonic acidemia (MMA, MIM 251000) is a form of metabolic acidosis caused by a defect in propionate metabolism at the step of conversion of methylmalonyl-CoA to succinyl-CoA [1]. MMA is caused by a functional defect in the methylmalonyl-CoA mutase (MCM, EC 5.4.99.2), which converts L-methylmalonyl-CoA to succinyl-CoA, due either to a mutation of its gene (*mut*$^0$ or *mut*) or to a defect in metabolism of its cofactor, deoxyadenosyl cobalamin (*cbl A-H*) [1–3].

So far, over 50 disease-causing *mut* mutations have been identified, along with many polymorphisms [4–10]. Several *mut* mutations have been identified in Japanese patients [11,12], but relatively little has been done in the rest of Asia. Here, we have identified the first Thai case of *mut*$^0$ MMA to be confirmed at the molecular level, identifying two novel mutations.

## Patient and methods

### *Patient*

The female infant of unrelated Thai parents presented at age 2 days with tachypnea and lethargy. Laboratory data indicated severe metabolic acidosis with a very wide anion gap. Urine organic acid analysis by GC-MS [13] revealed marked elevation of methylmalonic acid.

### *Enzyme assay*

Leukocytes were extracted and MCM activity assayed as previously described with reduced reagent volumes [14,15]. The $K_m$ of methylmalonyl-CoA was determined in 105 μM deoxyadenosyl cobalamin, while the $K_m$ of deoxyadenosyl cobalamin was determined in 380 μM methylmalonyl-CoA.

### *MCM gene amplification and sequencing*

Total RNA was extracted from the leukocytes using a Qiagen blood RNA kit (Qiagen GmbH, Hilden,

* Corresponding author. Fax: +66-2-248-0375.
*E-mail address:* scjsv@mahidol.ac.th (J. Svasti).
[1] Authors contributed equally and should be considered first authors.

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Germany). The MCM cDNAs were amplified by RT-PCR, as previously described [4], and directly sequenced. For analysis of the allelic segregation, the cDNA were cloned into pGEMT vectors (Promega, Madison, WI) and sequenced. Genomic DNA was prepared using a Qiamp DNA minikit (Qiagen). To analyze the 1048delT mutation, exon 5 was PCR amplified using the For987 and Rev1131 primers [4]. Exons 8 and 9 were amplified with the flanking primers, Ex8F (5′-GAAAATACATC ATAACCAGAGCA-3′) and Ex8R (5′-TAATACACA CCTCATGCTGTTG-3′) for exon 8, and Ex9F (5′-CA TCAGGGTCTAATCTCTTGAT-3′) and Ex9R (5′-TC ACATGGTTTACAGGATCAAC-3′) for exon 9, to detect the A499T and G544X mutations, respectively. The 1048delT mutation was confirmed by cleavage of the exon 5 PCR product with *Alu*I restriction endonuclease (New England BioLabs, Beverly, MA).

*MCM expression in Escherichia coli*

The mRNA of the proband and a normal control were reverse-transcribed and PCR amplified using *Pfu* polymerase (Promega) and the primers MCMF-*Nco*I (5′-AT TTCCATGGTACACCAGCAACAGCCCCT-3′) and MCMR-*Sac*I (5′-ATTTGAGCTCTCTCTTCTTTGAT CATAACTA-3′) to add *Nco*I and *Sac*I sites, cloned into these sites in pET32a and pET23d (Novagen, Madison, WI), and sequenced. To isolate the A499T and G544X mutations from other mutations and PCR errors, nucleotides 1160–1741 containing these mutations were excised with *Bam*HI and *Nsi*I, and ligated into the corresponding sites in the MCM cDNA expression vector to create single mutant expression vectors. These constructs were used for protein expression, and the *E. coli* cell extracts assayed for MCM activity and protein content, as previously described [16].

## Results and discussion

The proband had typical clinical presentation and urine organic acid pattern of MMA. No MCM activity could be detected in leukocyte extracts from the proband, whereas activity was detected in all normal controls ($121 \pm 50$ pmol succinyl-CoA produced/min/mg protein) and in the parents (78 and 52 pmol/min/mg for the mother and father, respectively).

The proband's cDNA had three heterozygous nucleotide changes: 1571G > A (A499T), 1706G > T, and 1707G > A, with the later two on the same allele to give 1706_1707delGGinsTA (G544X), and one heterozygous single base deletion, 1048delT. The previously described polymorphism H532R [5] was homozygous in all cDNA from this family and in three Thai controls. None of the new mutations were clearly detected in the mRNA from the parents, but genomic DNA sequence showed that the mother was heterozygous for the 1048delT and A499T mutations, while the father was heterozygous for the G544X mutation. The presence of the 1048delT mutation in the proband and her mother, but not the father, could be confirmed by PCR amplification of exon 5, followed by *Alu*I digest. The mutation eliminates an *Alu*I site, resulting in only approximately half the PCR product being digested in the mother and patient. The inability to detect the mutations in the parents' mRNA may indicate that the mutant mRNAs are less stable than the normal MCM mRNA. The 1048delT deletion causes a frameshift at Ala324, resulting in a change of the next eight residues from GRRLWAHL to VEDSGLT (stop), so both new mutations result in premature stop codons. The instability of MCM mRNA with premature stop codons has been noted in the past for other mutations resulting in premature stop codons [11].

The A499T change in this patient would not have any effect, since it comes after the 1048delT frameshift, but it is unclear whether it might affect other patients. Berger et al. [17] reported it in association with the mutation IVS8 + 3a > g, which apparently caused a high frequency of incorrect splicing. They suggested that the A499T mutation had no affect, since the position is not evolutionarily conserved. This mutation did not seem to affect the splicing, since no mis-spliced mRNA was detected here. MCM specific activities in extracts of *E. coli* expressing thioredoxin–MCM fusion proteins with normal MCM cDNA and A499T cDNA were high and similar ($8.53 \times 10^3$ and $8.11 \times 10^3$ pmol succinyl-CoA/mg/min), while those with the G544X mutation had no activity. Expression of MCM without the N-terminal thioredoxin fusion protein gave similar results. The $K_m$ values of the normal and A499T MCM for the cofactor, deoxyadenosyl-cobalamin, in the presence of 0.38 mM substrate were 0.26 and 0.19 μM, respectively, while $K_m$ values for the substrate, methylmalonyl-CoA, were 0.13 and 0.14 mM, respectively. Thus, the A499T MCM enzyme appeared normal in terms of binding cofactor and substrate and catalyzing the mutase reaction. Analysis of 100 Thai controls found this polymorphism represented 8.0% of the alleles (16 of 200 chromosomes).

The patient appeared to be a compound heterozygote for two new mutations, 1048delT and G544X. Both mutants are expected to produce a protein with a truncated MCM domain and no cobalamin-binding domain [18,19], so no MCM activity is expected. The A499T polymorphism, however, seemed to produce a normal enzyme in the recombinant system, and was found to be a frequent allele in the normal Thai population.

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