Original article

Clinical features and *MUT* gene mutation spectrum in Chinese patients with isolated methylmalonic acidemia: identification of ten novel allelic variants

Lian-Shu Han, Zhuo Huang, Feng Han, Jun Ye, Wen-Juan Qiu, Hui-Wen Zhang, Yu Wang, Zhu-Wen Gong, Xue-Fan Gu

Shanghai, China

Background: **This study aims to study *MUT* gene mutation spectrum in Chinese patients with isolated methylmalonic academia (MMA) and their clinical features for the potential genotype-phenotype correlation.**

Methods: **Forty-three patients were diagnosed with isolated MMA by elevated blood propionylcarnitine, propionylcarnitine to acetylcarnitine ratio, and urine methylmalonate without hyperhomocysteinemia. The *MUT* gene was amplified by polymerase chain reaction and directly sequenced. Those patients with at least one variant allele were included. The novel missense mutations were assessed by bioinformatic analysis and screened against alleles sequenced from 50 control participants.**

Results: **Among the 43 patients, 38 had typical clinical presentations, and the majority (30/38) experienced early-onset MMA. Eight patients died and seven were lost to follow-up. Twenty patients had poor outcomes and eight showed normal development. The 43 identified *MUT* gene mutations had at least one variant allele, whereas 35 had two mutant alleles. Of the 33 mutations reported before, eight recurrent mutations were identified in 32 patients, and c.729_730insTT (p.D244Lfs*39) was the most common (12/78) in the mutant alleles. Of the 10 novel mutations, six were missense mutations and four were premature termination codon mutations. The six novel missense mutations seemed to be pathogenic.**

Conclusions: **A total of 10 novel *MUT* mutations were detected in the Chinese population. c.729_730insTT (p.D244Lfs*39) was the most frequent mutation. A genotype-phenotype correlation could not be found, but the genotypic characterization indicated the need of genetic counseling for MMA patients and early prenatal diagnoses for high-risk families.**

World J Pediatr 2015;11(4):358-365

Key words: **methylmalonic acidemia; missense mutation; *MUT* gene**

Introduction

Methylmalonic acidemia (MMA) is an autosomal recessive inherited disorder of organic acid metabolism caused by a defect in the isomerization of methylmalonyl-CoA to succinyl-CoA. The reaction is catalyzed by the mitochondrial enzyme methylmalonyl-CoA mutase (MCM, EC 5.4.99.2) with adenosylcobalamin (Adocbl) as a cofactor. Isolated MMA is biochemically characterized by the abnormal accumulation of methylmalonic acid in the urine and other metabolites without hyperhomocysteinemia. MMA can be caused either by abnormal MCM activity [termed mut type MMA (OMIM 251000)] or a defect in the synthesis of its cofactor Adocbl, which forms part of the cbl complementation groups, namely cblA (OMIM 607481), cblB (OMIM 607568), and cblD-variant 2 (OMIM 606169).[1] Patients with mut MMA can be divided into two subtypes: mut^0 with completely defective MCM in fibroblasts and mut^- with residual MCM activity in the presence of high concentrations of cobalamin.

The clinical presentation and biochemical parameters of affected patients vary and can include poor feeding, failure to thrive, recurrent vomiting, lethargy, muscular hypotonia, seizures, mental and motor retardation or degeneration, metabolic ketoacidosis, and hyperammonemia.[2,3] Most of patients present early in

Author Affiliations: Department of Pediatric Endocrinology and Genetic Metabolism, Xinhua Hospital, Shanghai Institute for Pediatric Research, Shanghai Jiaotong University School of Medicine, Shanghai, China (Han LS, Huang Z, Han F, Ye J, Qiu WJ, Zhang HW, Wang Y, Gong ZW, Gu XF)

Corresponding Author: Xue-Fan Gu, Department of Pediatric Endocrinology and Genetic Metabolism, Xinhua Hospital, Shanghai Institute for Pediatric Research, Shanghai Jiaotong University School of Medicine, Shanghai 200092, China (Tel: +86-21-25076453; Fax: +86-21-65791316; Email: gu_xuefan@163.com)

doi: 10.1007/s12519-015-0043-1

©Children's Hospital, Zhejiang University School of Medicine, China and Springer-Verlag Berlin Heidelberg 2015. All rights reserved.

their life with life-threatening acute metabolic crises resulting in multi-organ damage, and without appropriate intervention for MMA they can be fatal. Though the diagnosis is rapid and treatment is proper, the long-term outcome remains disappointing due to neurological and renal complications.[4-7] Hörster et al[8] demonstrated that mut⁻subtype, a later onset of symptoms or response to cobalamin, is associated with more favorable outcomes.

MCM is a *MUT* gene-encoded mitochondrial matrix homodimer. The *MUT* gene, located on chromosome 6p12.3 and containing 13 exons, encodes 750 amino acids. The first 32 residues form a mitochondrial leader sequence, directing the entry of the precursor protein into the mitochondria, but are removed to form a mature subunit of 718 amino acids. The two mature subunits assemble to form a homodimer. The N-terminal extended segment (residues 33-87) is involved in the interaction between the subunits; residues 88-422 and 578-750 form the two main functional domains, namely, the substrate-binding domain and Adocbl-binding domain, which are attached by a long linker region (residues 423-577).[9,10] Since the *MUT* gene was mapped and cloned 20 years ago, almost 250 mutations have been detected in various populations. Most of the mutations were identified only in isolated cases, whereas a few were recurrent and accounted for 10%-20% of *MUT* gene mutations.[1] Among the recurrent mutations, some are specific to certain ethnicities and spread by founder effects, such as c.2150G>T (p.G717V) in the Black population; c.655A>T (p.N219Y) in Caucasians; c.349G>T (p.E117*), c.385+5G>A, and c.1481T>A (p.L494*) in the Japanese; c.729_730insTT (p.D244Lfs*39), c.1280G>A (p.G427D), and c.1630_1631delGGinsTA (p.G544*) in the Chinese.[11-14] However, some have been identified in patients of different ethnic backgrounds, and many of them occur in locations involving a CpG dinucleotide, such as codon 228 containing a CpG dinucleotide. Two mutations at this site have been reported, p.R228Q and p.R228*, and the latter has been identified in Mexican, Vietnamese, non-Hispanic Caucasian, and Black patients.[15] In the present study we observed the clinical features and *MUT* gene mutation spectrum in 43 patients, identifying 43 mutations in this cohort of Chinese patients, including 10 novel mutations. The possible effects of novel missense mutations on MCM presentation were interpreted.

Methods

Patients and diagnosis

This study included 43 patients (27 males and 16 females) diagnosed with isolated MMA, who had at least one variant allele of the *MUT* gene and were followed up at the hospital from December 2003 to March 2014. Fifty children without MMA from the health examination center served as controls. Written informed consent was obtained from the parents or legal guardians of study participants. The study was approved by the Ethics Committee of Xinhua Hospital (approval ID: XHEC-D-2014-035).

All of the patients were diagnosed with isolated MMA based on their clinical features, elevated blood levels of propionylcarnitine (C3), and the C3 to acetylcarnitine (C2) ratio (C3/C2) as measured by tandem mass spectrometry (MS/MS; AB Sciex, API 4000, Foster City, California, United State), and elevated urine methylmalonate levels as detected by gas chromatography-mass spectrometry (GC-MS; Shimadzu Limited, QP2010, Koyto, Japan). Some patients who were recruited from the newborn screening did not have any clinical symptoms. Furthermore, the plasma total homocysteine level was measured to exclude the possibility of intracellular cobalamin metabolism disorders as well as transport defects and nutritional abnormalities;[1] the levels were found to be in the normal range. Responsiveness to vitamin B12 was monitored in all patients as follows: hydroxocobalamin (1 mg/d, intramuscular) was administered for five days in combination with a low-protein diet (a formula free of isoleucine, valine, methionine, and threonine); a significant (more than 50%) reduction in the blood C3/C2 ratio and urine level of methylmalonate was considered to indicate vitamin B12 responsiveness.[1]

MUT gene mutation detection

Genomic DNA was extracted from peripheral blood leukocytes obtained from the patients and their parents using the TIANamp Blood DNA Kit (TIANGEN Biotech, Beijing, China). The exonic and flanking intronic regions of the *MUT* gene were amplified by polymerase chain reaction (PCR) from genomic DNA templates using previously described primers.[15] The PCR products were sequenced directly using forward and reverse primers on an ABI 3700 sequencer (Applied Biosystems, Foster City, California, United State). Mutation analysis was performed using the normal human *MUT* sequence as a reference (obtained from GenBank; accession number: NM_000255.3). The nucleotide numbering reflects cDNA numbering, with c.1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to the recommendations of Dunnen and Antonarakis.[16] The initiation codon is codon 1. All detected mutations were confirmed in the patients' parents.

Verification and assessment of the pathogenicity of novel variants

The Human Gene Mutation Database (http://www.hgmd.org/), Leiden Variation Open Database (http://www.genomed.org/lovd/), and the National Center

Original article

for Biotechnology dbSNP database (http://www.ncbi.nlm.nih.gov/SNP) were used to identify whether the mutations had been reported previously.[17] The novel identified variants were subsequently verified and screened in 100 alleles from the control individuals to distinguish mutations from polymorphisms. The prediction of splicing patterns was performed with the software GENSCAN (http://genes.mit.edu./GENSCAN.html). Novel nucleotide change resulting in nonsense, frame-shift, or abnormal splicing was considered as a pathogenic mutation. Novel missense variants were considered pathogenic when predicted to be deleterious by two algorithms, SIFT (http://sift.jcvi.org/) and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2). The evolutionary conservation across species of the affected amino acid was confirmed by multiple sequence alignments in Coelomata (*Homo sapiens*, *Pan troglodytes*, *Macaca mulatta*, *Canis lupus*, *Bos taurus*, *Mus musculus*, *Rattus norvegicus*, *Gallus gallus*, *Danio rerio*, and *Caenorhabditis elegans*), with the use of HomoloGene (http://www.ncbi.nlm.nih.gov/homologene/20097) and ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The amino acid substitutions were mapped onto a three-dimensional (3D) model to assess the potential impact of mutations on the structure of MCM protein, using DeepView software (version 4.1) and a 1.95-Å resolution human MCM crystallographic structure (PDB ID: 2XIJ, previously reported by Froese et al).[10]

Results

Clinical and biochemical findings

In total, the 43 patients had at least one variant allele of the *MUT* gene detected. In this series, 34 patients were recruited after being suspected of inherited metabolism disorders and nine patients were identified via newborn screening. The patients came from 14 provinces of the Mainland of China, representing 42 families. Ten patients (23.3%) came from Shandong province, eight (18.6%) from Henan provence, seven (16.3%) from Zhejiang province, and five (11.6%) from Jiangsu province. Another 13 patients came from other ten provinces. The patients were classified according to the onset of clinical symptoms and vitamin B12 responsiveness. Five patients were identified at the routine newborn screening before symptoms were observed, and the remaining 38 patients exhibited typical symptoms of MMA, of which 30 (78.9%) patients experienced early-onset of symptoms (before 1 year of age) [20 (66.7%) of these experienced neonatal-onset]. The initial symptoms frequently included recurrent vomiting (28 patients, 73.7%), disorders of consciousness (28 patients, 73.7%), poor feeding (23 patients, 60.5%), respiratory distress (20 patients, 52.6%), failure to thrive (20 patients, 52.6%), dystonia (20 patients, 52.6%), mental retardation or degeneration (14 patients, 36.8%), and convulsion (12 patients, 31.6%). Hyperammonemia, metabolic acidosis, and lactic acidosis were common. The median blood level of C3 was 13.56 (4.71-81.02) μmol/L, and the median ratio of C3 to C2 was 0.85 (0.36-2.89). The median urine level of methylmalonate was 598.96 (18.50-2976.25) mmol/mol creatinine. According to the results of vitamin B12 loading test, four patients (P3, P8, P11, and P26) were diagnosed with vitamin B12-responsive MMA (Table).

Eight of the 43 patients died during an acute metabolic crisis, seven were lost to follow-up, and 28 were alive at the end of the study in March 2014. Most surviving patients exhibited poor growth and neurological complications such as mental retardation and motor dysfunction of various degrees, but none had renal or other organ complications. Four patients (P3, P26, P35, and P36) identified during newborn screening remained asymptomatic, of whom patients P3 and P26 were vitamin B12 responsive and patients P35 and P36 were identical twins. Another four patients (P17, P19, P33, and P37) exhibited normal development without further symptoms, of whom patients P17 and P33 experienced neonatal onset of symptoms and patients P19 and P37 experienced late-onset of symptoms (Table).

Mutation analysis

A total of 43 different *MUT* gene mutations were identified, of which ten were firstly reported. Mutations were found on both alleles in 35 patients (81.4%). Among these patients, one (P9) carried two variants in the paternal allele and one mutation in the maternal allele. However, only one variant was detected in eight patients (P26, P27, P31, P33, P37, P38, P39, and P43). Mutations were found in all coding exons except for exon 7, and exon 3 had the highest number of mutations. The majority of the missense mutations (23/27) were located in the main functional domains, 17 in the substrate-binding barrel and six in the Adocbl-binding domain. The other four missense mutations were detected in the linker domain.

Of the previously reported mutations, 21 (63.6%) were missense mutations, seven (21.2%) were nonsense mutations, three (9.1%) were small insertions, duplications or indels, and two (6.1%) were consensus splice site mutations (Supplementary Table 1).[18-28] Among them, although p.G703R was reported previously, the nucleotide substitution (c.2107G>C) was different from that identified in this study (c.2107G>A).[27] Because the amino acid substitutions at the protein level were the same, this mutation was not classified as a novel mutation. Most of these mutations

were found in one family, whereas eight were identified in more than one unrelated patient, including three missense mutations [c.323G>A (p.R108H), c.1106G>A (p.R369H), and c.1280G>A (p.G427D)], two nonsense mutations [c.1741C>T (p.R581*) and c.2179C>T(p.R727*)], one small insertion [c.729_730insTT (p.D244Lfs*39)], one indel [c.1630_1631delGGinsTA (p.G544*)], and one splice site mutation (c.1677-1G>A).

The 10 novel mutations consisted of 6 missense mutations [c.419T>C (p.L140P), c.421G>A (p.A141T), c.482G>T (p.G161V), c.1540C>A (p.Q514K), c.1790T>G (p.I597R), and c.2168G>A (p.G723D)], 1 nonsense mutation [c.1777G>T (p.E593*)], two small duplications [c.626dupC (p.K210*) and c.810_811dupGG (p.A271Gfs*12)], and one small deletion [c.2106delA (p.V704*)] (Supplementary Table 1). All the novel mutations, aside from the [c.482G>T (p.G161V)] mutation, were found as a heterozygous change (Table). None of the 6 missense mutations was found in the 100 alleles from the control individuals.

Novel missense mutations and their potential pathogenic effects

The bioinformatic characteristics of the six novel mutations are summarized in Supplementary Table 2. Multiple sequence alignment revealed that all the residues of

Table. Identification of the MUT gene mutations in 43 patients with methylmalonic acidemia

Patient*	Sex	Newborn screening	Onset at diagnosis	Age of onset	Responsiveness to vitamin B12	Outcome†	Genotypes‡
Patients in a homozygous state							
P1	M	No	Yes	10 mon	No	Alive at 11 mon; unfavorable	c.[323G>A]; [323G>A]
P2	M	Yes	Yes	Neonatal	No	Died at 3 mon	c.**[482G>T]; [482G>T]**
P3	F	Yes	No	-	Yes	Alive at 4 mon; asymptomatic	c.[599T>C]; [599T>C]
P4	M	Yes	Yes	Neonatal	No	Alive at 2.8 y; unfavorable	c.[729_730insTT]; [729_730insTT]
P5	F	No	Yes	1.6 y	No	Unkown	c.[1106G>A]; [1106G>A]
Patients in a compound heterozygous/heterozygous state							
P6	F	No	Yes	1 mon	No	Unkown	c.[68C>G]; [323G>A]
P7	M	No	Yes	Neonatal	No	Died at 33 d	c.[103C>T]; [494A>G]
P8	M	No	Yes	8 mon	Yes	Unkown	c.[278G>A]; [567T>G]
P9	M	No	Yes	Neonatal	No	Alive at 1.8 y; unfavorable	c.[322C>T]; [323G>A; **2168G>A**]§
P10	M	No	Yes	6.4 y	No	Unkown	c.[323G>A]; [729_730insTT]
P11	F	No	Yes	9 mon	Yes	Died at 2 y	c.[323G>A]; [1514T>C]
P12	F	No	Yes	11 mon	No	Alive at 1.5 y; unfavorable	c.[323G>A]; [2179C>T]
P13	M	No	Yes	7 mon	No	Unkown	c.[**419T>C**]; [424A>G]
P14	M	No	Yes	2.4 y	No	Unkown	c.[**421G>A**]; [**810_811dupGG**]
P15	M	No	Yes	Neonatal	No	Died at 6 mon	c.[433G>A]; [1677-1G>A]
P16	M	No	Yes	Neonatal	No	Alive at 2.4 y; unfavorable	c.[454C>T]; [1280G>A]
P17	M	No	Yes	Neonatal	No	Alive at 6.5 y; favorable	c.[554C>T]; [2062G>T]
P18	M	No	Yes	Neonatal	No	Died at 5 mon	c.[**626dupC**]; [1531C>T]
P19	F	No	Yes	1.8 y	No	Alive at 2.4 y; favorable	c.[655A>G]; [729_730insTT]
P20	F	No	Yes	Neonatal	No	Alive at 1 y; unfavorable	c.[729_730insTT]; [1105C>T]
P21	M	No	Yes	Neonatal	No	Died at 9 mon	c.[729_730insTT]; [1106G>A]
P22	F	No	Yes	11 mon	No	Alive at 1.3 y; unfavorable	c.[729_730insTT]; [1106G>A]
P23	F	No	Yes	1.8 y	No	Alive at 3 y; unfavorable	c.[729_730insTT]; [1295A>C]
P24	M	No	Yes	Neonatal	No	Alive at 6.5 y; unfavorable	c.[729_730insTT]; [**1540C>A**]
P25	M	Yes	Yes	Neonatal	No	Died at 3 mon	c.[729_730insTT]; [1630_1631delGGinsTA]
P26	F	Yes	No	-	Yes	Alive at 6.6 y; asymptomatic	c.[729_730insTT]
P27	M	No	Yes	Neonatal	No	Alive at 1.8 y; unfavorable	c.[729_730insTT]
P28	M	No	Yes	Neonatal	No	Alive at 6.8 y; unfavorable	c.[754-1G>A]; [1061C>T]
P29	M	Yes	Yes	Neonatal	No	Alive at 3.2 y; unfavorable	c.[755dupA]; [1741C>T]
P30	M	No	Yes	Neonatal	No	Died at 1 mon	c.[925T>G]; [1630_1631delGGinsTA]
P31	M	No	Yes	Neonatal	No	Alive at 3.7 y; unfavorable	c.[1106G>A]
P32	M	No	Yes	Neonatal	No	Alive at 1.8 y; unfavorable	c.[1280G>A]; [1677-1G>A]
P33	M	No	Yes	Neonatal	No	Alive at 1.8 y; favorable	c.[1280G>A]
P34	M	No	Yes	2 mon	No	Alive at 5.5 y; unfavorable	c.[1630_1631delGGinsTA]; [1880A>G]
P35	F	Yes	No	-	No	Alive at 9.3 y; asymptomatic	c.[1630_1631delGGinsTA]; [1943G>A]
P36	F	Yes	No	-	No	Alive at 9.3 y; asymptomatic	c.[1630_1631delGGinsTA]; [1943G>A]
P37	F	No	Yes	3 y	No	Alive at 5.8 y; favorable	c.[1677-1G>A]
P38	M	No	Yes	Neonatal	No	Alive at 1.5 y; unfavorable	c.[1741C>T]
P39	F	No	Yes	1 y	No	Alive at 3.4 y; unfavorable	c.[1741C>T]
P40	F	No	Yes	8 mon	No	Alive at 2.1 y; unfavorable	c.[**1777G>T**]; [2179C>T]
P41	M	No	Yes	8.8 y	No	Alive at 10.3 y; unfavorable	c.[**1790T>G**]; [**2106delA**]
P42	F	No	Yes	10 mon	No	Alive at 2.3 y; unfavorable	c.[2080C>T]; [2179C>T]
P43	M	Yes	No	2 y	No	Alive at 2.3 y; unfavorable	c.[2107G>A]

*: P35 and P36 are identical twins; †: At the end of the study in March 2014; ‡: Novel mutations are in bold; §: Three variants were found: two in one allele and one in another allele. Favorable: normal cognitive and motor development without further episodes of metabolic decompensation. Unfavorable: poor neurological outcome such as mental retardation and motor dysfunction in different degrees; Unkown: lost to follow-up; "-": having no onset yet; M: male; F: female.

interest but 1 (Ile597) were strictly conserved across all 10 species studied. Nine of the 10 residues were isoleucine and one was methionine at position 597. All the native amino acid residues involved were located in the significant secondary structure. The PolyPhen-2 server predicted c.1790T>G (p.I597R) to be potentially damaging to the structure of MCM, and the other mutations were predicted to be probably damaging. All the mutations were expected to affect protein function according to the SIFT server.

The conceivable 3D conformations of wild-type and mutant MCM proteins are illustrated in Fig. In the case of c.419T>C (p.L140P), Leu was replaced by the smaller amino acid Pro (Fig. A). For c.421G>A (p.A141T), the wild-type non-polar hydrophobic amino acid Ala was replaced by a larger polar hydrophilic amino acid, Thr, and structural studies suggest that the mutation might create a new side chain-side chain hydrogen bond between Thr141 and Asp139 (Fig. B). For c.482G>T (p.G161V), the wild-type residue Gly161 was the smallest and simplest amino acid, whereas the larger mutant residue Val161 with a bulky side chain probably resulted in steric clashes with the neighboring residues (Fig. C). For c.1540C>A (p.Q514K), the wild-type residue Glu (a polar neutral amino acid) was replaced by Lys (a basic amino acid with a positive charge), and this substitution was predicted to inhibit hydrogen bonds with Asp165 and Asp169, form a new hydrogen bond with another residue, and create a steric interference in the protein (Fig. D). For c.1790T>G (p.I597R), the wild-type residue Ile597 (a non-polar hydrophobic amino acid) was replaced with Arg (a bigger basic hydrophilic amino acid with positive charge), and structural analysis suggested that this mutation may cause the formation of a new hydrogen bond with Gly592 and cause steric clashes with other residues (Fig. E). For c.2168G>A (p.G723D), the wild-type residue Gly723 (a neutral amino acid) was replaced with Asp (a negatively charged acidic amino acid) with a bulky side chain, which is likely to form a new side chain hydrogen bond with Tyr710 (Fig. F).

Discussion

MMA is the most common form of organic acidemia in China.[2,14,29] The mut type MMA is responsible for a large number of cases of isolated MMA. This study reports the clinical features and MUT gene mutation spectrum in a largest cohort of patients with isolated MMA from the mainland of China. The clinical and biochemical findings of our patients were in agreement with those reported previously.[2,3] Patients

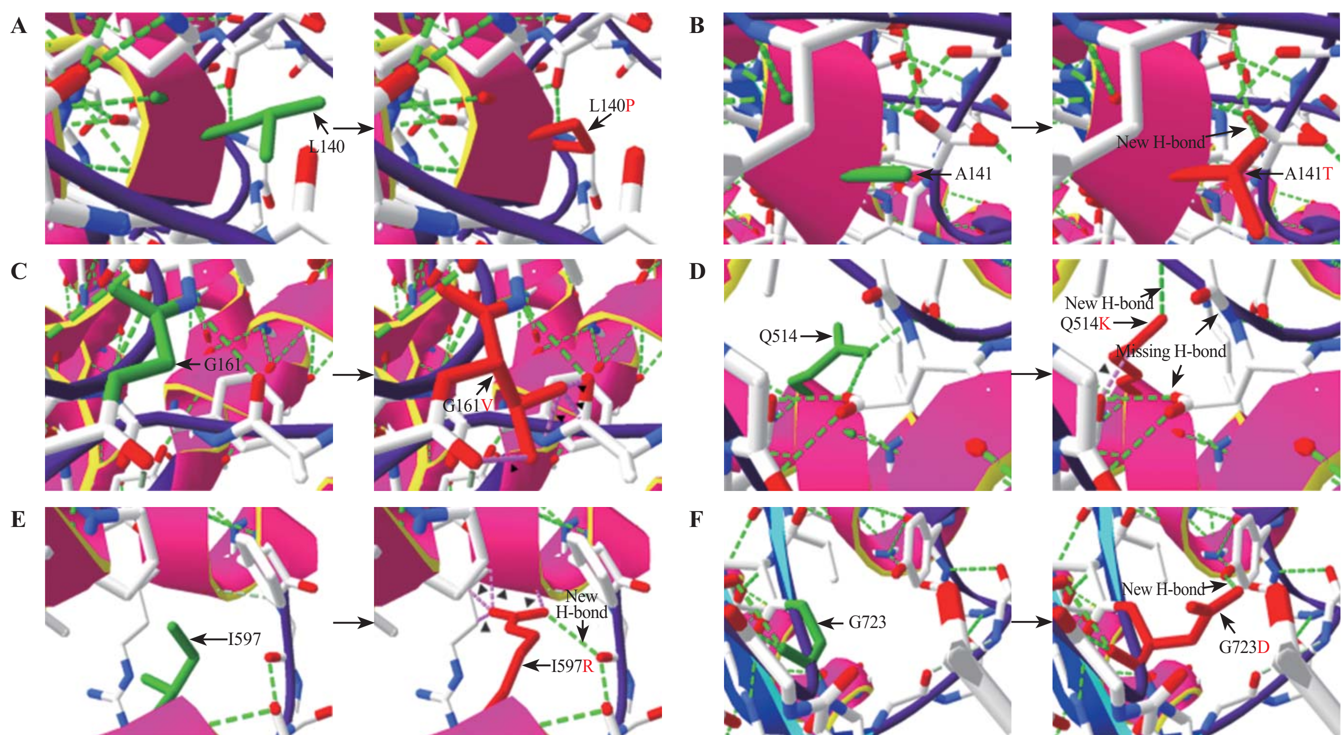

Fig. Three-dimensional models of human MCM bearing wild-type (green) and mutant amino acid residue (red) of the six novel missense mutations: L140P (**A**), A141T (**B**), G161V (**C**), Q514K (**D**), I597R (**E**), and G723D (**F**). The hydrogen bonds (H-bonds) are in green dotted line. The pink dotted lines with little black triangle represent the steric clash between atoms.

diagnosed from the neonatal screening and treated at the presymptomatic stage seem to have better outcomes. In addition, a few of our patients had increased blood C3 and C3/C2 but mildly abnormal urinary methylmalonate levels (e.g., 18.5, 47.9, and 51.9 mmol/mol creatinine in patients P3, P23, and P41, respectively). These findings indicate that clinicians should not rely solely on the urinary methylmalonate level for the diagnosis of MMA, so as to prevent misdiagnosis. The results of this study strongly indicate that molecular analysis can serve as a gold standard for the confirmation of the diagnosis.

Of 35 patients who carried two mutant alleles, five were homozygous and 30 were compound heterozygous for the mutations. In another eight patients, only one heterozygous change was detected, suggesting that the second mutation was located in noncoding or regulatory regions of *MUT* or involved a large gene deletion. As patient skin fibroblasts were unavailable, however, enzymatic and complementation studies were not performed to rule out the other types of isolated MMA, and thus the eight patients may harbor mutations in both alleles of the genes encoding cblA, cblB, or cblD-variant 2.

Of the 43 different mutations identified by the molecular analysis of the *MUT* gene, 33 were reported previously and 10 were novel mutations. In the mutations reported previously, 8 were recurrent mutations identified in 74.4% of our probands (32 patients). Four frequently identified mutations were not reported to be population specific, but rather involve CpG dinucleotides, namely c.323G>A (p.R108H), c.1106G>A (p.R369H), c.1741C>T (p.R581*), and c.2179C>T (p.R727*). Six additional previously reported CpG dinucleotide mutations [c.278G>A (p.R93H), c.322C>T (p.R108C), c.454C>T (p.R152*), c.1105C>T (p.R369C), c.1531C>T (p.R511*), and c.2080C>T (p.R694W)] were found. This study also confirms the high frequency of the three probable founder mutations in the Chinese population, namely, c.729_730insTT (p.D244Lfs*39), c.1280G>A (p.G427D), and c.1630_1631delGGinsTA (p.G544*).[14] Eighteen patients possessed at least one of these mutations, representing 23.3% (20/86) of the alleles tested, and 25.6% (20/78) of the total disease alleles. Further, c.729_730insTT (p.D244Lfs*39) was the most common among the three mutations and accounted for 15.4% (12/78) of all the mutant alleles. Six other mutations were detected more than once in Chinese unrelated families [c.103C>T (p.Q35*), c.424A>G (p.T142A), c.494A>G (p.D165G), c.599T>C (p.I200T), c.755dupA (p.H252Qfs*6), and c.1295A>C (p.E432A)].[14,30]

With respect to genotype–phenotype correlations, the limited number of patients included in this study, the frequency of compound heterozygotes and lack of enzymatic studies make it difficult to assess the precise relationship between gene mutations and clinical manifestations. Only four of the previously reported mutations were found in the homozygous state in this study. Patient P1 was homozygous for c.323G>A (p.R108H), exhibited symptoms at 10 months of age, and experienced severely retarded psychomotor development. In the previously reported patients, the c.323G>A (p.R108H) mutation was only detected in the compound heterozygous state and associated with both the mut^0 and mut$^-$ phenotype.[12,15,23,28] Patient P3, identified in newborn screening with mildly increased urinary methylmalonate excretion (18.5 mmol/mol creatinine), was homozygous for c.599T>C mutation. This mutation was previously described in a late-onset patient heterozygous for this mutation and c.103C>T,[14] and P3 remained asymptomatic with B12 treatment. Patient P4 was homozygous for c.729_730insTT (p.D244Lfs*39), who experienced neonatal-onset symptoms and both mental and motor retardation. Patient P5 was homozygous for c.1106G>A (p.R369H), a known mut^0 mutation,[28] and developed the typical symptoms at 1.6 years of age. However, most patients homozygous for c.1106G>A were reported to experience neonatal onset.[15,28]

Individuals with two protein truncating mutations are likely to have a severe phenotype.[15] Four patients (P18, P25, P29, and P40) were identified with two truncating mutations; three patients experienced neonatal onset and one exhibited symptoms at eight months of age, two died during the follow-up, and two had mental retardation. However, there is no evidence indicating that individuals with at least one missense mutation have a milder and later presentation. In addition, the mut$^-$ mutation usually plays a dominant role when it presents with a mut^0 mutation,[23] which may partly explain the relatively good clinical condition of twin sisters P35 and P36, who possessed mut^0 mutation c.1630_1631delGGinsTA and mut$^-$ mutation c.1943G>A. Furthermore, interallelic complementation complicated the prediction of potential genotype-enzymatic subtype correlations as patients can have a mut$^-$ enzymatic subtype in the compound state but a mut^0 enzymatic subtype in the homozygous state.[15,19]

A total of 10 novel mutations were identified in eight patients, one homozygous and seven compound heterozygous. Four of the novel mutations were found in four patients, and were predicted to produce truncated peptides. Two patients with late onset symptoms were compound heterozygous for the truncated mutation and a novel missense mutation, respectively (c.421G>A and c.810_811dupGG; c.1790T>G and c.2106delA). The mutation c.626dupC (p.K210*) along with the known mutation c.1531C>T (p.R511*) was detected in patient P18, who had neonatal presentations and

died at the age of 5 months. The mutation c.1777G>T (p.E593*) was found along with the known mutation c.2179C>T (p.R727*) in patient P40, who manifested symptoms at the age of 8 months and was alive with mental retardation. We hypothesize that patient P18 and P40 belong to the mut^0 enzymatic subtype as they each carry two null mutations predicted to be early degraded by nonsense-mediated mRNA decay[14] and patients previously reported to be homozygous for their known mutations were mut^0.[15,23] The other six novel mutations were missense mutations found in six patients, and all occurred in segments highly conserved across the tested species, suggesting that these residues are crucial for the stability and function of MCM. The functional prediction scores of our novel missense mutations supported this assumption. Three novel missense mutations were located within the substrate-binding barrel, the binding site of coenzyme A. The mutation c.419T>C (p.L140P) along with the known mutation c.424A>G (p.T142A) was detected in patient P13, who showed initial symptoms at the age of 7 months. The c.419T>C (p.L140P) mutation, localized in a small helix, may influence the conformation of this secondary structure of the enzyme. The mutation c.421G>A (p.A141T) was found along with a novel frame-shift mutation c.810_811dupGG (p.A271Gfs*12) in a late-onset patient (P14). This missense mutation was mapped to the same helix as c.419T>C (p.L140P), which may reduce hydrophobic interactions in the core of the protein. The mutation c.482G>T (p.G161V) was detected in the homozygous state in patient P2, who was symptomatic at newborn screening and died at 3 months of age. Although the Gly161 residue localized in a buried site in a β turn lacks a CpG dinucleotide, two mutations have been previously reported at this site (p.G161* and p.G161R) and are associated with mut^0 phenotype.[15,31] The substitution valine may disturb the local structure and cause instability. The severe clinical phenotype as well as the functional and structural prediction suggests that c.482G>T (p.G161V) is likely to be a mut^0 mutation. Only one novel missense mutation c.1540C>A (p.Q514K) is located in the linker domain. This mutation along with c.729_730insTT (p.D244Lfs*39) was found in a neonatal-onset patient (P24), who showed mental retardation at the time of the last follow-up. The c.1540C>A (p.Q514K) mutation located in a helix introduces a charge in a buried residue, which is predicted to affect protein folding. Two other novel mutations are located within the cofactor-binding domain. The mutation c.1790T>G (p.I597R) along with the novel mutation c.2106delA (p.V704*) was detected in a late-onset patient (P41) with unfavorable outcome, whose urinary methylmalonate level was mildly elevated (51.9 mmol/mol creatinine) during the first attack. Residue 597 is located in a helix; the mutation is predicted to be unsuitable for the hydrophobic interactions in the core of the protein and is likely to alter protein folding. The mutation c.2168G>A (p.G723D) is located on the surface of the protein, and the substitution introduces charge, adds bulk, and decreases flexibility, which is likely to affect protein folding and stability. Patient P9 exhibited severe clinical manifestation in the neonatal period, and then delayed mental and motor development. This patient was compound heterozygous for c.322C>T (p.R108C) in the maternal allele and both c.323G>A (p.R108H) and c.2168G>A (p.G723D) in the paternal allele. p.R108C and p.R108H have been reported in patients with a range of ethnic backgrounds due to the CpG dinucleotide located in codon 108. All patients homozygous for c.322C>T were mut^0, while the c.323G>A (p.R108H) mutation was only detected in compound heterozygous level and may express both mut^0 and mut^- phenotype.[12,15,23,28] The c.2168G>A (p.G723D) mutation was predicted to be pathogenic, but it was difficult to understand the pathogenic effect of this mutation in patient P9 with two known causative allele mutations. The findings indicate that all the native amino acid residues involved are highly/fully conserved and mapped to significant secondary structural elements, and that the substitutions of different size, charge, or hydrophobicity are likely to impact on folding and/or structural stability of MCM, rather than directly influencing catalysis.

In conclusion, the 43 different mutations of the *MUT* gene were detected in 43 Chinese patients with isolated MMA. These mutations included eight recurrent mutations and ten novel mutations thought to be pathogenic based on the bioinformatic analysis. However, no definite relationship was found between the mutations and the clinical manifestations of the patients. Nonetheless, the genotypic characterization indicates the need for genetic counseling for MMA patients and early prenatal diagnosis for high-risk families.

Acknowledgements

We thank all the patients, non-MMA controls, and their families for their participation in this study. We also express our gratitude to all of the pediatricians who provided patient data and blood samples.

Funding: This study was supported by grants from the National Key Technology R&D Program (2012BAI09B04) and the Special Basic Work of Science and Technology (2014FY110700).

Competing interest: The authors declare that they have no competing interests.

Contributors: Han LS and Huang Z contributed equally to this work. Han LS wrote the first draft of this paper. All authors contributed to the intellectual content and approved the final version. Gu XF is the guarantor.

References

1 Fowler B, Leonard JV, Baumgartner MR. Causes of and diagnostic approach to methylmalonic acidurias. J Inherit Metab Dis 2008;31:350-360.
2 Yang Y, Sun F, Song J, Hasegawa Y, Yamaguchi S, Zhang Y, et al. Clinical and biochemical studies on Chinese patients with methylmalonic aciduria. J Child Neurol 2006;21:1020-1024.
3 Van Gosen L. Organic acidemias: a methylmalonic and propionic focus. J Pediatr Nurs 2008;23:225-233.
4 Knerr I, Weinhold N, Vockley J, Gibson KM. Advances and challenges in the treatment of branched-chain amino/keto acid metabolic defects. J Inherit Metab Dis 2012;35:29-40.
5 Hörster F, Baumgartner MR, Viardot C, Suormala T, Burgard P, Fowler B, et al. Long-term outcome in methylmalonic acidurias is influenced by the underlying defect (mut^0, mut^-, cblA, cblB). Pediatr Res 2007;62:225-230.
6 Cosson MA, Benoist JF, Touati G, Déchaux M, Royer N, Grandin L, et al. Long-term outcome in methylmalonic aciduria: a series of 30 French patients. Mol Genet Metab 2009;97:172-178.
7 Baumgartner MR, Hörster F, Dionisi-Vici C, Haliloglu G, Karall D, Chapman KA, et al. Proposed guidelines for the diagnosis and management of methylmalonic and propionic acidemia. Orphanet J Rare Dis 2014;9:130.
8 Hörster F, Garbade SF, Zwickler T, Aydin HI, Bodamer OA, Burlina AB, et al. Prediction of outcome in isolated methylmalonic acidurias: combined use of clinical and biochemical parameters. J Inherit Metab Dis 2009;32:630-639.
9 Thomä NH, Leadlay PF. Homology modeling of human methylmalonyl-CoA mutase: a structural basis for point mutations causing methylmalonic aciduria. Protein Sci 1996;5:1922-1927.
10 Froese DS, Kochan G, Muniz JR, Wu X, Gileadi C, Ugochukwu E, et al. Structures of the human GTPase MMAA and vitamin B12-dependent methylmalonyl-CoA mutase and insight into their complex formation. J Biol Chem 2010;285:38204-38213.
11 Adjalla CE, Hosack AR, Matiaszuk NV, Rosenblatt DS. A common mutation among blacks with mut^- methylmalonic aciduria. Hum Mutat 1998;Suppl 1:S248-S250.
12 Acquaviva C, Benoist JF, Callebaut I, Guffon N, Ogier de Baulny H, Touati G, et al. N219Y, a new frequent mutation among mut^0 forms of methylmalonic acidemia in Caucasian patients. Eur J Hum Genet 2001;9:577-582.
13 Sakamoto O, Ohura T, Matsubara Y, Takayanagi M, Tsuchiya S. Mutation and haplotype analyses of the *MUT* gene in Japanese patients with methylmalonic acidemia. J Hum Genet 2007;52:48-55.
14 Liu MY, Liu TT, Yang YL, Chang YC, Fan YL, Lee SF, et al. Mutation Profile of the *MUT* Gene in Chinese Methylmalonic Aciduria Patients. JIMD Rep 2012;6:55-64.
15 Worgan LC, Niles K, Tirone JC, Hofmann A, Verner A, Sammak A, et al. Spectrum of mutations in mut methylmalonic acidemia and identification of a common Hispanic mutation and haplotype. Hum Mutat 2006;27:31-43.
16 den Dunnen JT, Antonarakis SE. Mutation nomenclature extensions and suggestions to describe complex mutations: a discussion. Hum Mutat 2000;15:7-12.
17 Vatanavicharn N, Champattanachai V, Liammongkolkul S, Sawangareetrakul P, Keeratichamroen S, Ketudat Cairns JR, et al. Clinical and molecular findings in Thai patients with isolated methylmalonic acidemia. Mol Genet Metab 2012;106:424-429.
18 Wang F, Han L, Ye J, Qiu W, Zhang Y, Gao X, et al. Analysis of the *MUT* gene mutations in patients with methylmalonic academia. Zhonghua Yi Xue Yi Chuan Xue Za Zhi 2009;26:485-489. [In Chinese]
19 Raff ML, Crane AM, Jansen R, Ledley FD, Rosenblatt DS. Genetic characterization of a *MUT* locus mutation discriminating heterogeneity in mut^0 and mut^- methylmalonic aciduria by interallelic complementation. J Clin Invest 1991;87:203-207.
20 Martínez MA, Rincón A, Desviat LR, Merinero B, Ugarte M, Pérez B. Genetic analysis of three genes causing isolated methylmalonic acidemia: identification of 21 novel allelic variants. Mol Genet Metab 2005;84:317-325.
21 Jung JW, Hwang IT, Park JE, Lee EH, Ryu KH, Kim SH, et al. Mutation analysis of the *MCM* gene in Korean patients with MMA. Mol Genet Metab 2005;84:367-370.
22 Janata J, Kogekar N, Fenton WAl. Expression and kinetic characterization of methylmalonyl-CoA mutase from patients with the mut^- phenotype: evidence for naturally occurring interallelic complementation. Hum Mol Genet 1997;6:1457-1464.
23 Acquaviva C, Benoist JF, Pereira S, Callebaut I, Koskas T, Porquet D, et al. Molecular basis of methylmalonyl-CoA mutase apoenzyme defect in 40 European patients affected by mut^o and mut^- forms of methylmalonic acidemia: identification of 29 novel mutations in the *MUT* gene. Hum Mutat 2005;25:167-176.
24 Champattanachai V, Ketudat Cairns JR, Shotelersuk V, Keeratichamroen S, Sawangareetrakul P, Srisomsap C, et al. Novel mutations in a Thai patient with methylmalonic acidemia. Mol Genet Metab 2003;79:300-302.
25 Fuchshuber A, Mucha B, Baumgartner ER, Vollmer M, Hildebrandt F. mut^0 methylmalonic acidemia: Eleven novel mutations of the methylmalonyl CoA mutase including a deletion-insertion mutation. Hum Mutat 2000;16:179.
26 Crane AM, Ledley FD. Clustering of mutations in methylmalonyl CoA mutase associated with mut^- methylmalonic acidemia. Am J Hum Genet 1994;55:42-50.
27 Qureshi AA, Crane AM, Matiaszuk NV, Rezvani I, Ledley FD, Rosenblatt DS. Cloning and expression of mutations demonstrating intragenic complementation in mut^0 methylmalonic aciduria. J Clin Invest 1994;93:1812-1819.
28 Lempp TJ, Suormala T, Siegenthaler R, Baumgartner ER, Fowler B, Steinmann B, et al. Mutation and biochemical analysis of 19 probands with mut^0 and 13 with mut^- methylmalonic aciduria: identification of seven novel mutations. Mol Genet Metab 2007;90:284-290.
29 Han L, Han F, Ye J, Qiu W, Zhang H, Gao X, et al. Spectrum analysis of common inherited metabolic diseases in Chinese patients screened and diagnosed by tandem mass spectrometry. J Clin Lab Anal 2015;29:162-168.
30 Yi Q, Lv J, Tian F, Wei H, Ning Q, Luo X. Clinical characteristics and gene mutation analysis of methylmalonic aciduria. J Huazhong Univ Sci Technolog Med Sci 2011;31:384-389.
31 Filippi L, Gozzini E, Cavicchi C, Morrone A, Fiorini P, Donzelli G, et al. Insulin-resistant hyperglycaemia complicating neonatal onset of methylmalonic and propionic acidaemias. J Inherit Metab Dis 2009;32 Suppl 1:S179-S186.

Received April 19, 2015
Accepted after revision July 14, 2015

(Supplementary information is linked to the online version of the paper on the *World Jounal of Pediatrics* website)

Original article