Mutation Profile of the *MUT* Gene in Chinese Methylmalonic Aciduria Patients

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Abstract The *mut*-type methylmalonic aciduria (MMA, MIM 251000) is caused by a deficiency of mitochondrial methylmalonyl-CoA mutase (MCM, E.C. 5.4.99.2) activity, which results from defects in the *MUT* gene. To elucidate the mutation spectrum of the *MUT* gene in Chinese MMA

patients, 13 exons of the *MUT* gene, including untranslated regions, were analyzed by PCR-based sequencing for 42 unrelated Chinese MMA patients. All the 42 patients were found to have at least one *MUT* mutation. A total of 41 mutations were identified. Of these mutations, 20 were

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novel ones, including one nonsense mutation (c.103C>T), 12 missense mutations (c.316A>C, c.424A>G, c.494A>G, c.554C>T, c.599T>C, c.919T>C, c.1009T>C, c.1061C>T, c.1141G>A, c.1208G>A, c.1267G>A, and c.1295A>C), one duplication (c.755dupA), three small deletions (c.398_399delGA, c.1046_1058del, and c.1835delG), two mutations that might affect mRNA splicing (c.754-1G>A and c.1084-10A>G), and one major deletion. Among the mutations identified, the c.1280G>A (15.5%), c.729_730insTT (10.7%), c.1106G>A (4.8%), c.1630_1631GG>TA (4.8%), and c.2080C>T (4.8%) accounted for 40% of the diseased alleles. The c.1280G>A and c.729_730insTT mutations were found to be the most frequent mutations in Southern and Northern Chinese, respectively. The results of microsatellite analysis suggest that the spread of c.729_730insTT among the Northern Chinese and of c.1280G>A and c.1630_1631GG>TA among the Southern Chinese may have undergone founder effects. This mutation analysis of the gene responsible for *mut*-type MMA will help to provide a molecular diagnostic aid for differential diagnosis of MMA and could be applied for carrier detection and prenatal diagnosis among Chinese family at risk of *mut*-type MMA.

Abbreviation

MCM Methylmalonyl-CoA mutase

MMA Methylmalonic aciduria

Introduction

Methylmalonic aciduria (MMA) is a group of rare autosomal recessive disorders of methylmalonate and cobalamin metabolism and has an incidence estimated to vary from 1/50,000 to 1/80,000 (Horster and Hoffmann 2004). In the presence of cofactor adenosylcobalamin (AdoCbl), the mitochondrial enzyme methylmalonyl-CoA mutase (MCM; EC 5.4.99.2) catalyzes the isomerization of methylmalonyl-CoA to succinyl-CoA (Fowler et al. 2008). Defects either in the apoenzyme (*mut*-type MMA) or in biosynthesis of the cofactor impair MCM function and result in an accumulation of methylmalonate in the body fluids (Fowler et al. 2008). Some defects in cobalamin metabolism, such as *cbiC*, *cbiD*, and *cbiF*, affect both the biosynthesis of AdoCbl and methylcobalamin and are characterized by combined MMA and homocystinuria (Fowler et al. 2008). Abnormalities in AdoCbl synthesis only, namely, *cbiA*, *cbiB*, and *cbiD* variant 2, cause isolated MMA (Fowler et al. 2008). Patients with different etiologies have similar clinical manifestations, including lethargy, recurrent vomiting, failure to thrive, dehydration, respiratory distress, and hypotonia (Zwickler et al. 2008).

The treatment for MMA includes dietary protein restriction, L-carnitine supplementation, and oral antibiotics administration to reduce the production of propionate by intestinal bacteria (Zwickler et al. 2008). Supplementation of hydroxocobalamin or cyanocobalamin is used for cobalamin-responsive patients (Zwickler et al. 2008), and betaine supplementation is required for patients with combined MMA and homocystinuria in order to reduce the homocysteine levels and restore methionine (Rosenblatt et al. 1997). Therefore, differential diagnosis of the different types of MMA is important to the implementation of appropriate treatments for MMA patients.

The *mut*-type MMA (OMIM 251000) is caused by a defect in MCM apoenzyme, which is encoded by the nuclear *MUT* gene. The *MUT* gene is located on chromosome 6p21 and consists of 13 exons spanning over 35 kb. The human MCM precursor contains an N-terminal mitochondrial targeting sequence of 32 amino acids and two functional domains, a (β/α)₈ barrel (residues 88–422) substrate-binding site and a C-terminal ($\beta\alpha$)₅ B12-binding domain (residues 578–750). After entering the mitochondria and removal of the leader sequence, two identical subunits form the functional enzyme (Fowler et al. 2008).

Up to the present, near 200 mutations in the *MUT* gene have been identified in various populations and reported in the Human Gene Mutation Database (www.hgmd.cf.ac.uk) (Fowler et al. 2008), and most of them are private mutations. However, some recurrent mutations occur in the *MUT* gene, and these have been found across a range of ethnic backgrounds and include c.323G>A (p.R108H), c.682C>T (p.R228X), and c.1106G>A (p.R369H) (Worgan et al. 2006). In addition, ethnic-specific mutations have also been found, including c.2150G>T (p.G717V) in African-American patients; c.655A>T (p.N219Y) in Caucasian patients; c.322C>T (p.R108C) in Hispanic patients; c.349G>T (p.E117X), c.1481T>A (p.L494X), and c.385+5G>A in Japanese patients; and c.1280G>A (p.G427D) and c.1630_1631delGGinsTA (p.G544X) in Asian patients (Worgan et al. 2006).

MMA is the most common organic aciduria found in Chinese patients (Hori et al. 2005). Furthermore, *mut*-type MMA, with an incidence of about 1/100,000, is one of the most common organic aciduria disorders in Taiwan (Niu et al. 2010). In order to understand the molecular defects found in Chinese patients with MMA, we examined the spectrum of mutations in *MUT* gene from 42 Chinese MMA patients. A total of 41 mutations were identified in 42 patients, including 20 novel mutations and 21 previously reported mutations. Among these mutations, c.729_730insT and c.1280G>A were identified as the most frequent mutations in Northern and Southern Chinese, respectively.

Material and Methods

Subjects

The 42 unrelated Chinese patients with MMA (25 males and 17 females) were clinically diagnosed based on elevated urinary methylmalonic acid using gas chromatography/mass spectrometry (GC/MS). Samples from 22 Northern Chinese patients (collected from Beijing), 16 Southern Chinese patients (14 were collected from Taiwan, 1 from Shanghai, 1 from Beijing), three patients of unknown provinces (collected from Beijing), one patient with mixed background (from Taiwan), and their family members were collected. None of these patients were from consanguineous families. Among these 42 patients, 20 were symptomatic in the neonatal stage, nine were symptomatic within 1 year of birth, six presented at an age after 1 year, six were identified by neonatal screening programs (Niu et al. 2010), and the clinical information for the remaining one patient was unavailable.

Cultured fibroblast cell lines, lymphoblast cell line, or phytohemagglutinin (PHA)-stimulated leukocytes were obtained from 23 patients. These 23 patients with available cell lines were confirmed to be diagnosed as *mut*-type MMA by remarkably reduced or undetectable intracellular MCM activity in the cultured cell lines (Table 1). MCM activity was directly determined by measuring the conversion of L-methylmalonyl-CoA to succinyl-CoA using high-performance liquid chromatography (Kikuchi et al. 1989). For the remaining 19 patients, normal plasma total homocysteine (tHcy) levels were used for detection and thus rule out MMA combined with homocystinuria. However, confirmatory diagnosis could not be made in these 19 patients due to a lack of cultured cell lines. Therefore, an approach of direct mutational analysis of the *MUT* gene was used to aid in the study of molecular defects and to obtain confirmatory diagnosis in these patients. Samples from parents were available from 37 families.

In order to determine the allelic frequencies of any newly identified DNA sequence variations in the general population, DNA samples of 50 anonymous Southern Chinese controls from Taiwan and 53 anonymous Northern Chinese controls from Beijing were collected. The Ethics Committee of the Taipei Veterans General Hospital approved the study.

Mutation Analysis of the *MUT* Gene

Genomic DNA was extracted from peripheral leukocytes, cultured fibroblasts, or lymphoblasts using standard methods. The method for genomic DNA isolated from dried blood spot sample has been described previously (Hong

et al. 1999; Liu et al. 1998). All 13 exons of the *MUT* gene, including coding and non-coding exons, and exon-intron boundary sequences were PCR-amplified and directly sequenced by thermocycle sequencing using the BigDye[®] terminator 3.1 sequencing kit (Applied Biosystems, Foster City, CA, USA) following analysis by an ABI 3730XL DNA Analyzer (Applied Biosystems). The sequences were then explored using the Polyphred/Phrap/Consed system (www.phrap.com) (Gordon et al. 1998; Nickerson et al. 1997). The mutation nomenclature follows the recommendations from den Dunnen and Antonarakis (den Dunnen and Antonarakis 2000) using GenBank accession no. NM_000255.2 as the reference sequence. For newly identified sequence variations, the alterations were confirmed by sequencing an independent PCR product. Disease-causing mutations were confirmed by studying the prevalence of these mutations among the normal Chinese controls, linkage analysis in the families, recurrence of the mutations, and/or the conservation of the affected amino acid. To evaluate the effect of the sequence variations on pre-mRNA splicing, the ESE finder method (<http://rulai.cshl.edu/tools/ESE>) (Cartegni et al. 2003) was used to predict possible exonic splicing enhancers (ESEs) and effect of any variations on splicing.

Short Tandem Repeat Analysis

One microsatellite marker, namely, D6S269, was studied. This STR is located 221 kb downstream of the *MUT* gene. This polymorphic marker was analyzed using PCR amplification with one of 5' fluorescent-labeled primers. Fragments were separated on autosequencer, and data were analyzed using GeneScan and Genotyper software (Applied Biosystems). Primers used for the D6S269 are Forward: FAM labeled-CCTTGCTCATGGTTTACAA and Reverse: CAGAAAGACATGGTAGAAGAGG (<http://www.ncbi.nlm.nih.gov/genome/sts/sts.cgi?uid=34004>). The allelic frequency of each allele was determined among the general Chinese controls, the patients, and their parents. Fisher's exact test was used to evaluate the difference between the distribution of the normal and mutant alleles with a *p* value < 0.01 considered to be significant.

Results and Discussion

In this study, sequence analysis of genomic DNA from 42 unrelated Chinese MMA families was used to identify the disease-causing mutations in the *MUT* gene. In total, two mutations in the *MUT* gene were identified in 37 patients, while in five patients, only a single mutation was identified (Table 1). MCM activity was measured for 23 *mut*-type

Table 1 Mutations identified in the *MUT* gene of the 42 Chinese MMA patients

Patient ID	Gender	Age of onset	MCM ^a	Mutation 1	Mutation 2	Origin
M125	F	3 months	ND	c.91C>T	c.729_730insTT	N
M105	M	1.5 years	ND	c.103C>T	c.599T>C	N
M006	M	4 months	0.1 ^b	c.316A>C	c.1630_1631GG>TA	N/S
M132	F	5 months	ND	c.322C>G	c.1267G>A	N
M094	M	1 month	ND	c.323G>A	c.424A>G	N
M118	M	5 days	<0.05 ^c	c.323G>A	c.1741C>T	S
M069	F	Neonate	<0.05 ^c	c.398_399delGA	c.755dupA	N
M155	M	3 days	<0.05 ^c	c.454C>T	c.1280G>A	S
M113	M	1.3 years	ND	c.494A>G	c.494A>G	N
M161	M	3 days	ND	c.554C>T	c.2062G>T	S
M037	M	22 h	<0.05 ^c	c.682C>T	NF	N
M003	M	Neonate	<0.05 ^b	c.683G>A	c.1046_1058del	S
M086	F	Neonate	ND	c.729_730insTT	c.1009T>C	N
M119	M	6 months	ND	c.729_730insTT	c.1084-10A>G	N
M142	M	1.2 years	ND	c.729_730insTT	c.1106G>A	N
M145	M	3 days	ND	c.729_730insTT	c.1718T>C	N
M070	F	Neonate	<0.05 ^c	c.729_730insTT	c.1880A>G	U
M091	F	5 months	ND	c.729_730insTT	c.2080C>T	N
M100	M	7–8 months	<0.05 ^c	c.729_730insTT	c.914T>C	N
M025	M	Neonate	<0.05 ^c	c.729_730insTT	c.970G>A	N
M144	M	Neonate	ND	c.754-1G>A	c.1061C>T	U
M126	F	NS	<0.05 ^c	c.755dupA	c.1280G>A	S
M136	F	1 day	ND	c.914T>C	c.1677-1G>A	N
M114	F	3.5 years	0.84 ^c	c.919T>C	c.1280G>A	S
M174	M	NS	<0.05 ^d	c.982C>T	c.982C>T	S
M028	M	No record	ND	c.1084-10A>G	c.1106G>A	N
M015	M	40 days	<0.05 ^b	c.1106G>A	c.1741C>T	N
M096	F	Neonate	ND	c.1106G>A	NF	U
M065	M	2.5 years	<0.05 ^c	c.1141G>A	c.2080C>T	N
M080	M	7 days	ND	c.1208G>A	NF	N
M117	F	NS	0.44 ^c	c.1280G>A	c.1280G>A	S
M121	F	NS	<0.05 ^c	c.1280G>A	c.1280G>A	S
M007	F	6 days	0.26 ^b	c.1280G>A	c.1630_1631GG>TA	S
M018	F	5 days	<0.05 ^d	c.1280G>A	c.1677-1G>A	S
M115	M	NS	<0.05 ^c	c.1280G>A	c.1677-1G>A	S
M034	M	3 months	ND	c.1280G>A	c.1835delG	N
M005	M	3 days	<0.05 ^b	c.1280G>A	NF	S
M173	F	NS	<0.05 ^d	c.1280G>A	Major deletion	S
M160	M	4 years	ND	c.1295A>C	c.1399C>T	N
M058	F	<1 week	<0.05 ^c	c.1630_1631GG>TA	c.1943G>A	S
M008	M	2–4 days	<0.05 ^b	c.1630_1631GG>TA	NF	S
M151	F	9 months	ND	c.2080C>T	c.2080C>T	N

ND not done, NS newborn screening, MCM methylmalonyl-CoA mutase, NF not found, S Southern Chinese, N Northern Chinese, U Chinese patient without information on subpopulation

^a Normal reference range: 1.6–15.7 nmol/min/mg protein in skin fibroblasts; 3.2–15.8 nmol/min/mg protein in lymphoblasts; 1.7–12.6 nmol/min/mg protein in PHA-stimulated leukocytes.

^b Skin fibroblasts

^c Lymphoblasts

^d PHA-stimulated leukocytes

MMA patients and at least one *MUT* mutation was identified in all 23 patients. A total of 41 mutations (Table 2), including 20 novel ones and 21 previously reported mutations, were identified in these 42 patients (Table 1). These included 24 missense mutations, eight nonsense mutations, one duplication, one insertion, three small deletions, three mutations that might affect the splicing, and one major deletion. Sequence analysis identified 94% of all disease alleles in these 42 patients (Table 1). Except for the major deletion, all other mutations were identified in exon 2–12 and in the exon-intron boundaries. In five patients (M005, M008, M037, M080, and M096), only one heterozygous mutation was identified after scanning the 13 exons and the exon-intron boundary sequences of the *MUT* gene. Besides, we also sequenced the intronic region for which two splicing mutations have been reported, namely, c.1957-891 C>A and c.1957-898A>G (Perez et al. 2009; Rincon et al. 2007), and none of these two mutations were identified in the *MUT* gene of these patients. Among these five patients, patients M005, M008, and M037 were designated as *mut*-type MMA due to deficiencies of MCM activities using HPLC method with cultured fibroblasts or lymphoblasts. However, cell lines from M080 and M096 with MMA were unavailable, and information of enzymatic activities was absent; it did not rule out the possibility that M080 and M096 could be *cbIA*- or *cbIB*-type MMA. For these five patients, the second mutation might still be a large gene deletion, insertion, duplication, gene rearrangement, or in the non-coding regions. Further studies such as RT-PCR followed by cDNA sequencing are needed to elucidate the second defects in these subjects.

Novel Mutations

A total of 20 novel mutations were identified. The 20 novel mutations consisted of one nonsense mutation (c.103C>T [p.Q35X]), 12 missense mutations (c.316A>C [p.T106P], c.424A>G [p.T142A], c.494A>G [p.D165G], c.554C>T [p.S185F], c.599T>C [p.I200T], c.919T>C [p.F307L], c.1009T>C [p.F337L], c.1061C>T [p.S369C], c.1141G>A [p.G381R], c.1208G>A [p.R403Q], c.1267G>A [p.D423N], and c.1295A>C [p.E432A]), one duplication (c.755dupA), three small deletions (c.398_399delGA, c.1046_1058del, and c.1835delG), two mutations that might affect splicing (c.754-1G>A and c.1084-10A>G), and one major deletion (Table 2). None of these mutations were found in any members of the Southern Chinese (50 individuals) or Northern Chinese (53 individuals) control populations.

The duplication (c.755dupA) was identified in two unrelated families, whereas the three small deletions (c.398_399delGA, c.1046_1058del13, and c.1835delG)

were seen in three unrelated families (Table 1). These frameshift mutations are predicted to result in premature translational stop codons.

Two patients from unrelated families were both heterozygous for a C>T transition at position 103 in exon 2 (Table 1). The c.103C>T (p.Q35X) mutation is a nonsense mutation and is predicted to result in the substitution of a stop codon for a glutamine residue at codon 35.

Two novel mutations, namely, c.754-1G>A and c.1084-10A>G (Table 2), are predicted to affect splicing. The c.754-1G>A mutation was identified in one patient at position c.754-1 in intron 3, which involves an alteration of the acceptor consensus splice site and is predicted to disrupt the processing of *MUT* transcripts. The other mutation that might affect splicing (c.1084-10A>G) was identified in two unrelated families at position c.1084-10 in the intron 5. The c.1084-10A>G change is predicted to create a cryptic 3' splice site using ESE finder 3.0.

The remaining 12 mutations, namely, c.316A>C (p.T106P), c.424A>G (p.T142A), c.494A>G (p.D165G), c.554C>T (p.S185F), c.599T>C (p.I200T), c.919T>C (p.F307L), c.1009T>C (p.F337L), c.1061C>T (p.S354F), c.1141G>A (p.G381R), c.1208G>A (p.R403Q), c.1267G>A (p.D423N), and c.1295A>C (p.E432A) are missense mutations (Table 2) and were identified in 17 unrelated families (Table 1). Among the 12 mutations, ten (c.316A>C, c.424A>G, c.494A>G, c.554C>T, c.599T>C, c.919T>C, c.1009T>C, c.1061C>T, c.1141G>A, and c.1208G>A) are located in the N-terminal ($\beta\alpha$)₈ barrel substrate-binding site region of the enzyme, while two others are located in the linker region (c.1267G>A and c.1295A>C). Most of the amino acid residues (11/12) that are affected are conserved in bilateral orthologs (*Homo sapiens*, *Pan troglodytes*, *Canis lupus*, *Bos taurus*, *Mus musculus*, *Rattus norvegicus*, *Gallus gallus*, *Danio rerio*, *Caenorhabditis elegans*, HomoloGene:20097. Gene conserved in Bilateria, NCBI, data not shown). One missense mutation, namely, c.599T>C (p.I200T), is structurally conserved (isoleucine and valine) across these orthologs. In addition, the c.1009T>C alteration is predicted to create a SRP55 protein recognition site using ESE finder and might affect the splicing of exon 5. It should be interesting to confirm such an effect of c.1009T>C on cDNA level.

Mutation analysis based on the PCR sequencing showed that patient M173 was homozygous for the c.1280G>A mutation. However, the results of linkage analysis showed that the c.1280G>A mutation was only present in the *MUT* gene of the father and was not present in the mother (Supplementary Fig. 1). Sequence analysis of exon 3 showed that the father was heterozygous for the SNP c.636G>A (chromosome 6: 49,425,521, Build 37.2

Table 2 The *MUT* mutations, their predicted effect on the protein, and their allelic frequencies among Southern and Northern Chinese *mut*-type MMA patients

Location	Mutation ^a			Allele No.				Frequency
	Nucleotide	Amino acid	Domain	S	N	U	Total	(%)
Exon 2	c.91C>T	p.R31X	ML	—	1	—	1	1.2
	c.103C>T	p.Q35X	NT	—	1	—	1	1.2
	c.316A>C	p.T106P	($\beta\alpha$) ₈	—	1	—	1	1.2
	c.322C>G	p.R108G	($\beta\alpha$) ₈	—	1	—	1	1.2
	c.323G>A	p.R108H	($\beta\alpha$) ₈	1	1	—	2	2.4
Exon 3	c.398_399delGA	p.G133VfsX6	($\beta\alpha$) ₈	—	1	—	1	1.2
	c.424A>G	p.T142A	($\beta\alpha$) ₈	—	1	—	1	1.2
	c.454C>T	p.R152X	($\beta\alpha$) ₈	1	—	—	1	1.2
	c.494A>G	p.D165G	($\beta\alpha$) ₈	—	2	—	2	2.4
	c.554C>T	p.S185F	($\beta\alpha$) ₈	—	1	—	1	1.2
	c.599T>C	p.I200T	($\beta\alpha$) ₈	—	1	—	1	1.2
	c.682C>T	p.R228X	($\beta\alpha$) ₈	—	1	—	1	1.2
	c.683G>A	p.R228Q	($\beta\alpha$) ₈	1	—	—	1	1.2
	c.729_730insTT	p.D244LfsX39	($\beta\alpha$) ₈	—	8 ^c	1	9	10.7
Intron 3	c.754-1G>A	Splice	($\beta\alpha$) ₈	—	—	1	1	1.2
Exon 4	c.755dupA	p.H252QfsX6	($\beta\alpha$) ₈	1	1	—	2	2.4
	c.914T>C	p.L305S	($\beta\alpha$) ₈	—	2	—	2	2.4
	c.919T>C	p.F307L	($\beta\alpha$) ₈	1	—	—	1	1.2
	c.970G>A	p.A324T	($\beta\alpha$) ₈	—	1	—	1	1.2
	c.982C>T	p.L328F	($\beta\alpha$) ₈	2	—	—	2	2.4
Exon 5	c.1009T>C	p.F337L	($\beta\alpha$) ₈	—	1	—	1	1.2
	c.1046_1058del	p.A349DfsX20	($\beta\alpha$) ₈	1	—	—	1	1.2
	c.1061C>T	p.S354F	($\beta\alpha$) ₈	—	1	—	1	1.2
Intron 5	c.1084-10A>G	Splice	Splice	—	2	—	2	2.4
Exon 6	c.1106G>A	p.R369H	($\beta\alpha$) ₈	—	3	1	4	4.8
	c.1141G>A	p.G381R	($\beta\alpha$) ₈	—	1	—	1	1.2
	c.1208G>A	p.R403G	($\beta\alpha$) ₈	—	1	—	1	1.2
	c.1267G>A	p.D423N	Linker	—	1	—	1	1.2
	c.1280G>A	p.G427D	Linker	12 ^b	1	—	13	15.5
	c.1295A>C	p.E432A	Linker	—	1	—	1	1.2
Exon 7	c.1399C>T	p.R467X	Linker	—	1	—	1	1.2
Exon 9	c.1630_1631GG>TA	p.G544X	Linker	4 ^c	—	—	4	4.8
Intron 9	c.1677-1G>A	Splice	Splice	2	1	—	3	3.5
Exon 10	c.1718T>C	p.F573S	Linker	—	1	—	1	1.2
	c.1741C>T	p.R581X	($\beta\alpha$) ₅	1	1	—	2	2.4
Exon 11	c.1835delG	p.G612VfsX8	($\beta\alpha$) ₅	—	1	—	1	1.2
	c.1880A>G	p.H627R	($\beta\alpha$) ₅	—	1	—	1	1.2
	c.1943G>A	p.G648D	($\beta\alpha$) ₅	1	—	—	1	1.2
Exon 12	c.2062G>T	p.E688X	($\beta\alpha$) ₅	—	1	—	1	1.2
	c.2080C>T	p.R694W	($\beta\alpha$) ₅	—	4	—	4	4.8
	Major deletion			1	—	—	1	1.2
	NF	Unknown		2	2	1	5	6.0
	Total allele No.			31	49	4	84	

ML mitochondrial leader peptide, NT N-terminal extended segment, ($\beta\alpha$)₈ substrate-binding ($\beta\alpha$)₈ domain, ($\beta\alpha$)₅ B12-binding ($\beta\alpha$)₅ domain, *Splice* predicted to cause splicing defect, *S* Southern Chinese, *N* Northern Chinese, *U* patients without available information on province

^a Mutations newly described in this study are shown in bold

^b Fisher's exact test, $p < 0.001$

^c Fisher's exact test, $p > 0.01$

reference sequence) and the mother was a c.636G homozygote, whereas the patient was a c.636G>A homozygote (Supplementary Fig. 1). In addition, the results of the STR analysis showed that the proband was homozygous for the 190 bp of D6S269 marker (chromosome 6:49,176,851–49,177,030, Build 37.2 reference sequence), the father was compound heterozygous for the 182-bp and 190-bp allele, while the mother was homozygous for the 178-bp allele. These results suggest a DNA segment at least 248 kb in length (from chromosome 6:49,176,851–49,425,521), spanning from the exon 3 of the *MUT* gene to the D6S269 marker, which might be deleted in the maternal chromosome 6. A genome-wide SNP array with an average spacing between SNPs of 2.4 kb was then performed in M173 and the parents. The SNP analysis revealed a homozygosity between rs12176541 (chromosome 6: 48,530,298, Build 37.2 reference sequence) and rs2635727 (chromosome 6: 50,820,940) for M173 and her mother (data not shown). Additional SNP analysis between rs12176541 and rs2635727 indicated that both M173 patient and her mother harbored a deletion across genomic sequence of 2.2 Mb between SNP rs2052800 (chromosome 6: 48,534,929) and rs6930924 (chromosome 6: 50,790,633, Supplementary Fig. 1). The precise breaking points of this major deletion, however, remain to be determined.

Recurrent Mutations

Fourteen mutations were identified in more than one unrelated patient (Table 1). Five previously reported mutations, namely, c.729_730insTT, c.1106G>A, c.1280G>A, c.1630_1631GG>TA, and c.2080C>T (Champattanachai et al. 2003; Crane and Ledley 1994; Janata et al. 1997; Merinero et al. 2008; Worgan et al. 2006), were relatively frequently observed in Chinese patients (Table 2). Among these five mutations, the c.1280G>A and c.729_730insTT have been reported in Asian patients (Worgan et al. 2006), the c.1630_1631GG>TA mutation has been reported in patients from Thai and Chinese ethnic backgrounds (Champattanachai et al. 2003; Worgan et al. 2006), and the c.1106G>A and c.2080C>A mutations have been frequently observed in multiple ethnic backgrounds (Lempp et al. 2007; Merinero et al. 2008; Worgan et al. 2006).

The c.729_730insTT and c.1280G>A mutation accounted for 10.7% and 15.5% of the total disease alleles in the 42 *mut*-type Chinese MMA patients, respectively. The other frequently observed mutations were c.1106G>A, c.1630_1631GG>TA, and c.2080C>T mutation, with each mutation accounting for 4.8% of the total disease alleles. Around 67% of Chinese *mut*-type patients carried at least one of these five mutations.

The distributions of these five mutations in Southern and Northern Chinese populations were different. Among the frequent mutations, most (12/13) of the c.1280G>A and all c.1630_1631GG>TA ($n = 4$) alleles were seen in Southern Chinese patients and accounted for 38.7% and 13% of the disease alleles in Southern Chinese patients, respectively (Table 2), whereas most of the c.729_730insTT (8/9) alleles and all four c.2080C>T and all three c.1106G>A alleles were found in Northern Chinese patients and accounted for 16.3%, 8.2%, and 6.1% of disease alleles identified in Northern Chinese patients, respectively. Among these common mutations, the c.729_730insTT, c.1280G>A, and c.1630_1631GG>TA mutations were not located at a mutation hot spot (Cooper and Youssoufian 1988) and seemed to segregate across subethnic populations, suggesting these three mutations might have undergone a founder effect in the Chinese population.

STR Analysis of Microsatellite Marker D6S269

In order to study the founder effect with respect to these three mutations, the allelic distribution of the STR marker D6S269 in normal populations and the patients was analyzed. The D6S269 marker is located downstream of the *MUT* gene and is informative in the Chinese population. As shown in Table 3, a total of eight different alleles, ranging from 178 bp to 192 bp, were detected from 50 anonymous Southern Chinese controls and 53 anonymous Northern Chinese controls. No statistic difference was observed for allelic frequencies between the normal Northern and Southern Chinese controls; thus, the allelic frequencies of the total normal controls ($n = 206$) were used when comparing the distribution of D6S269 alleles in the normal population with that of the patients. Among the normal Chinese controls, the 182-bp allele was the most common genotype. Defined by linkage analysis, the phase of the four c.1630_1631GG>TA, five c.729_730insTT, and 13 c.1280G>A alleles was found to be closely linked to the 190-bp allele of the D6S269 marker, which is a relatively less frequent (8%) genotype in the normal population (Fisher's exact test, p value < 0.0001, Table 3). The results indicated that there was strong linkage disequilibrium between these three mutations and the 190-bp allele of D6S269, which suggests that spread of the c.729_730insTT among Northern Chinese and transmission of the c.1280G>A and c.1630_1631GG>TA mutations among Southern Chinese might have been influenced by separate founder effects.

Phenotype and Genotype

Among the 42 probands, 14 of them died, three were lost during follow-up, and 25 patients were alive at ages ranging from 1.5 months to 11 years. Most of the patients who were

Table 3 Allelic frequencies of the D6S269 marker among the general Chinese population and Chinese patients carrying the frequent mutations

Allele size	Controls ^a			Patients		
	Northern	Southern	Total	c.729_730insTT	c.1280G>A	c.1630_1631GG>TA
(bp)	N (%)	N (%)	N (%)	N ^b (%)	N ^b (%)	N ^b (%)
178	4 (3.8)	10 (10.0)	14 (6.80)	0 (0.0)	0 (0.0)	0 (0.0)
180	18 (17.0)	23 (23.0)	41 (19.90)	0 (0.0)	0 (0.0)	0 (0.0)
182	66 (62.3)	55 (55.0)	121 (58.73)	0 (0.0)	0 (0.0)	0 (0.0)
184	5 (4.7)	2 (2.0)	7 (3.40)	0 (0.0)	0 (0.0)	0 (0.0)
186	1 (0.9)	0 (0.0)	1 (0.49)	0 (0.0)	0 (0.0)	0 (0.0)
188	1 (0.9)	2 (2.0)	3 (1.46)	0 (0.0)	0 (0.0)	0 (0.0)
190	10 (9.4)	7 (7.0)	17 (8.25)	5 (100.0) ^c	13 (100.0) ^c	4 (100.0) ^c
192	1 (0.9)	1 (1.0)	2 (0.97)	0 (0.0)	0 (0.0)	0 (0.0)
Total allele no.	106	100	206	5	13	4

^a No statistic difference was found in the allelic distribution between Northern and Southern normal controls

^b Alleles of known phase

^c Significant different ($p < 0.0001$) vs. the normal Chinese controls (allele no. = 206)

still alive had significant developmental delay; this included the patients detected through the neonatal screening program who had undergone early extensive treatment (Niu et al. 2010). Three patients (M018 [c.1280G>A] + [c.1677-1G>A]), M114 ([c.1280G>A] + [c.919T>C]), and M160 ([c.1295A>C] + [c.1399C>T]) showed normal development. Patient M018 had received a liver transplantation and has a normal IQ at the age of 7 years.

It is difficult to interpret the relationship between phenotype and most of the mutations in the *MUT* gene identified in these Chinese patients because the majority of the patients were compound heterozygotes in this study. Only four mutations, namely, c.494A>G, c.982C>T, c.1280G>A, and c.2080G>A, were seen in homozygotes. Patient M113 with homozygous c.494A>G mutations presented with symptoms at the age of 1.3 years and was alive at the age of 3.5 years with mental retardation. Patient M174 harboring homozygous c.982C>T mutations was detected in neonatal screening program, had undetectable MCM activity, and showed symptoms at the neonatal stage. Although the patient underwent early continuous treatment, the patient had significant developmental delay. Patient M151 was homozygous for the c.2080C>T mutation and manifested symptoms at the age of 9 months; the patient was lost during follow-up. The clinical presentation results suggest that the c.494A>G and c.982C>T mutations are not mild mutations.

Of the 11 patients carrying the c.1280G>A mutation, two patients were homozygous and nine patients were heterozygous for c.1280G>A and other mutations. Ten of the 11 patients, including the two c.1280G>A homozygotes and eight heterozygotes, had symptoms during early

infancy (Table 1). In these eight patients who were heterozygous for the c.1280G>A mutation, the second mutations included a frameshift mutation (c.755dupA and c.1835delG), a nonsense mutation (c.454C>T, c.1630_1631GG>TA), a splice-site mutation (c.1677-1G>A), a major deletion, and a mutation in a non-coding region ($n = 1$); all of these second mutations can be suggested to be null mutations. These results indicate that an early-onset phenotype may be associated with this c.1280G>A missense mutation. The remaining one patient (M114) was exhibiting 26% of the lower limit for MCM activity in lymphoblasts (reference range: 3.2–15.8 nmol/min/mg protein) and was heterozygous for the c.1280G>A and c.919T>C missense mutations (Table 1). Patient M114 presented with symptoms at the age of 3.5 years. The propionylcarnitine (C3) level in neonatal dried blood spots (DBS) has never been determined before admission at age of 3.5 years because this patient was born before MMA newborn screening started. On admission, a newly collected DBS as well as the neonatal DBS were used to determine the blood C3 level. The MS/MS results of neonatal DBS showed a normal blood level of C3 (6.3 μ M; normal, <7 μ M) and slightly elevated C3/C2 ratio (0.368; normal, <0.2), while the DBS collected at disease onset showed that both C3 (7.41) and C3/C2 ratio (0.48) were elevated. This patient received conventional therapy, including dietary management and L-carnitine supplementation and had normal development at the age of 7 years. The milder phenotype of patient M114 might be associated with the c.919T>C mutation (p.F307L), which is predicted to substitute a leucine residue for a phenylalanine residue at the codon 307 in the substrate-binding domain. However,

more patients carrying the c.919T>C mutation are required in order to support this genotype-phenotype correlation.

Summary

In summary, a total of 20 novel mutations, including one nonsense, 12 missense, one duplication, three small deletions, two mutations that might affect the splicing, and one major deletion, as well as 21 previously reported mutations, have been detected in 42 Chinese *mut*-type MMA patients. Among these mutations, the c.729_730insTT mutation was identified in 16.3% of disease alleles from the Northern Chinese patients, while the c.1280G>A accounted for 38.7% of disease alleles from Southern Chinese patients. The spreading of the c.729_730insTT and c.1630_1631GG>TA and c.1280G>A mutations across various Chinese populations from different geographic regions might have involved a founder effect. Identification of these mutations in Chinese MMA patients will facilitate differential confirmatory diagnosis, which is important to the implementation of appropriate treatments. It will also aid carrier detection, genetic counseling, and subsequent prenatal diagnosis.

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Shu-Fen Lee	Conduct experiments
Yu-Ting Teng	Conduct experiments
Ying-Chen Chang	Conduct the molecular genetics study
Ya-Ling Fan	Biochemical and enzymatic studies
Szu-Hui Chiang	Collection of information
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Lian-Shu Han	Clinical diagnosis and collection of clinical information
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Statement of Competing Interest

The authors have nothing to declare.

Details of the Ethics Approval

Partial patients were retrospectively studied. This study was approved by the Ethics Committee of the Taipei Veterans General Hospital.

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