Eight novel *MUT* loss-of-function missense mutations in Chinese patients with isolated methylmalonic academia

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Background: **Isolated methylmalonic acidemia is a rare autosomal recessive metabolic disorder mostly caused by mutations in the methylmalonyl coenzyme A mutase (MCM) gene (*MUT*). This study aimed to verify whether missense mutations in *MUT* in Chinese patients affect the stability and enzymatic activity of MCM.**

Methods: **Eight Chinese patients were identified with novel mutations. Plasmids carrying the wild-type and mutated *MUT* cDNA were constructed and transfected into HEK293T cells for functional analyses. The expression and activity of MCM were determined by western blot and ultra-performance liquid chromatography, respectively.**

Results: **All patients had high levels of blood propionylcarnitine and urinary methylmalonyl acid. By the end of the study, two patients were lost to follow-up, three died, and three survived with mental retardation. Compared to the wild-type protein, the expression levels of all missense mutations of *in vitro* MCM protein were decreased ($P<0.05$) except those for I597R, and the MCM activity of the mutations was reduced in a permissive assay.**

Conclusion: **The missense mutations L140P, A141T, G161V, W309G, I505T, Q514K, I597R and G723D affected the stability and enzymatic activity of MCM, indicating that they had a disease-causing capacity.**

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Key words: **methylmalonic acid; methylmalonic acidemia; methylmalonyl-CoA mutation; missense mutation**

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Introduction

Methylmalonic acidemia (MMA), an autosomal recessive metabolic disorder, usually results in recurrent vomiting, metabolic acidosis, brain damage, and developmental delay.[1,2] Its incidence is 1/50 000-1/80 000 living births.[3] MMA is treated by dietary protein restriction or a special formula free of branched-chain amino acids, L-carnitine, and cobalamin (for cobalamin responsive patients).[3,4] However, metabolic decompensation cannot be completely avoided, and neurological sequalae are common.[5,6]

MMA is caused by a defect in the conversion of methylmalonyl-CoA to succinyl-CoA, a reaction catalyzed by methylmalonyl-CoA mutase (MCM) with adenosylcobalamin (AdoCbl) as a cofactor. Isolated MMA is mainly caused by mutations in the MCM gene (*MUT*), leading to decreased activity of MCM. These mutations may result in two subtypes identified in patients' fibroblasts: 1) mut^0, with undetectable MCM activity, or 2) mut^-, with residual MCM activity in the presence of high concentrations of cobalamin.[2] Up to the present, approximately 250 mutations in *MUT* have been reported in different populations. For example, c.2150G>T(p.G717V) is common in the Blacks,[7] c.655A>T(p.N219Y) in the Caucasians,[8] c.349G>T(p.E117*), c.385+5G>A or c.1481T>A(p.L494*) in Japanese,[9] and c.729_730insTT(p.D244Lfs*), c.1280G>A(p.G427D) or c.1630_1631delinsTA(p.G544*) in Chinese.[10] In our center, we found different *MUT* mutations in 43 patients with isolated MMA, of which c.729_730insTT mutation was the most common (15.4%).[11] But the following 6 missense mutations had not been reported: 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). In 2009, we identified two missense mutations [c.925T>G(p.W309G) and c.1514T>C(p.I505T)][12] and they were also not verified. Hence, the pathogenicity of the eight missense mutations is difficult to be speculated.[13] To assess the effect of the eight missense mutations, we conducted *in silico* assays and functional studies after transfection in HEK293T cells of pcDNA3.1-myc-hisC(+) plasmids carrying mutant and wild-type *MUT* cDNA. The results revealed that these mutations influenced the expression and activity of MCM.

Original article

Methods

Patients

In this study, eight missense mutations were detected in eight unrelated Chinese children with isolated MMA. The children included 7 males and 1 female, all of them were non-consanguineous. This study was approved by the Ethics Committee of Xinhua Hospital, Shanghai Institute for Pediatric Research, Shanghai Jiaotong University School of Medicine, Shanghai. The legal guardians of the children signed a written informed consent before the study.

DNA sequence analysis

DNA was extracted from leukocytes of peripheral blood using a Blood Genomic DNA Extraction Kit (Tiangen Biotech Co., Ltd., Beijing, China). The exonic and flanking intronic regions of *MUT* were amplified using reported primers for the 13 exons.[14] The PCR products were sent to the Beijing Genomics Institute (BGI, Shanghai, China) or Shanghai Sangon Biotech Co., Ltd. (China) for direct sequencing in both directions. The sequences were compared with the reference sequence in GenBank (NM_000255.3). The nucleotide numbering was performed according to the recommendations of den Dunen and Antonarakis.[15]

In silico assay

In silico assay was performed to predict the potential structural and functional effect of novel missense variants, such as SIFT (http://sift.jcvi.org/) and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/). The effect on protein conformation was studied by Swiss-PDBViewer 4.1, using a human three-dimensional crystal structure of MCM (PDB#2XIJ).

Construction of a wild-type plasmid

Full length *MUT* cDNA was obtained from normal human skin fibroblasts (Shanghai Institute for Pediatric Research, China) by RT-PCR using the forward primer 5'-GGTGGAATTCTGCAGATATCCACCATGTTAA GAGCTAAGAATC-3' and the reverse primer 5'-CGC CACTGTGCTGGATATTACAGATTGCTGCTTCTT TTCCA-3'. The resulting amplicon, which lacked the termination codon, was inserted into the mammalian expression vector pcDNA3.1-myc-hisC(+) (Shanghai Institute for Pediatric Research, China), which linearized beforehand by a recognition site for EcoRV (New England BioLabs Inc., USA), using a seamless cloning technique with the NovoRec PCR One-Step kit (Novoprotein Scientific Inc., Shanghai, China) (Fig. 1). The resulting wild-type plasmid was verified by direct sequencing.

Site-directed mutagenesis

The recombinant wild-type *MUT* cDNA plasmid was used as a template to construct the eight mutants by PCR site-

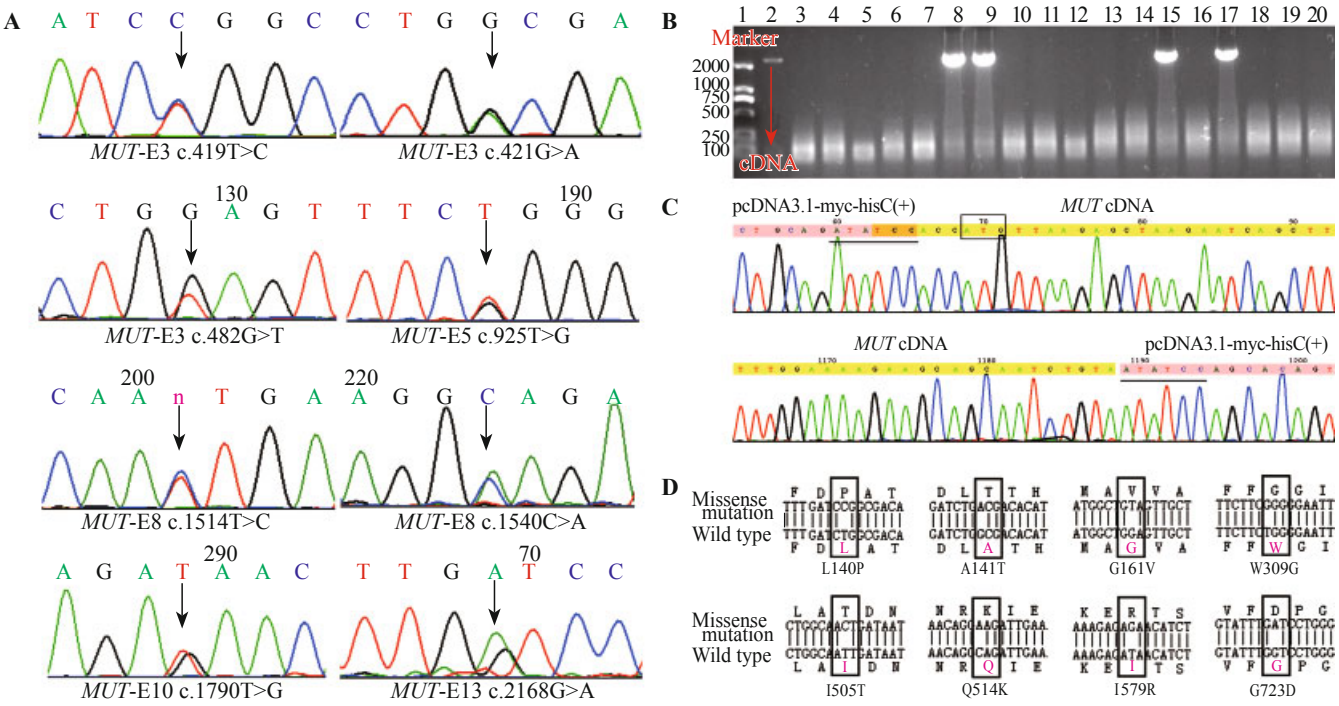

Fig. 1. Electropherograms of the eight *MUT* missense mutations and confirmation of the wild-type and eight mutant plasmids. **A**: Electropherograms of the eight *MUT* missense mutations; **B**: PCR products by specific primers for full-length *MUT* cDNA identified by agarose gel electrophoresis. Lane 1 is the 2 kb DNA ladder marker, and lane 2 is the template *MUT* cDNA. Lanes 3-20 are the "wild-type" plasmids to be confirmed; **C**: Electropherograms of the connection regions in recombinant plasmid; **D**: Sequence alignment of wild-type and mutated plasmids.

directed mutagenesis with the TransStart Fast Pfu PCR Super Mix (Beijing TransGen Biotech Co., Ltd.) for high-fidelity and long PCR, and DpnI (New England Biolabs Inc., USA) for digestion. The specific mutagenesis primers with partially overlapping forward and reverse primers, which were a little different from the conventional design,[16] are shown in Table 1. All constructed mutant plasmids were confirmed by direct sequencing.

Cell culture and transient transfection

HEK293T cells (Shanghai Institute for Pediatric Research, China) were grown in DMEM (GIBCO, Invitrogen Inc., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin (GIBCO, Invitrogen Inc., Carlsbad, CA, USA) in a 5% CO_2 atmosphere at 37°C. The cells were transfected with plasmids encoding wild-type or mutant MCM using lipofectamine 2000 (Invitrogen Inc., Carlsbad, CA, USA). The empty vector was transfected as a negative control. After 48-72 hours, the cells were harvested for further assays.

Western blot

Cell lysates were obtained by sonication in RIPA lysis buffer (BioTeke Corporation, Beijing, China). After centrifugation at 16 000×g for 20 minutes at 4°C, the resulting supernatant was used for an assessment of MCM expression. Protein supernatant was mixed with 5×SDS loading buffer (Beyotime, Jiangsu, China) and heated to 100°C for 10 minutes. Equal amounts of proteins (15 μg) were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare, Little Chanfont, UK). The membranes were blocked with 5% skimmed milk powder in a Tris-buffered saline solution with 0.1% Tween 20 for 2-4 hours at room temperature, incubated with polyclonal mouse anti-MCM antibody (Abcam, Cambridge, UK) or monoclonal mouse tubulin antibody (Beyotime, Beijing, China) overnight. Then, they were detected by incubation with goat anti-mouse antibody coupled to horseradish peroxidase (Jackson Immuno Research, West Grove, PA, USA) for 1 hour at room temperature, and visualized using the ECL reagent (Millipore Corp., Billerica, MA, USA) on a gel imaging system (Bio-Rad, CA, USA). Tubulin was used as a loading control. As *MUT* cDNA did not have a termination codon but a C-terminal Myc tag in the recombinant plasmid, monoclonal rabbit c-Myc antibody (Cell Signaling Technology, USA) was also used as primary antibodies for the western blot analysis.

MCM activity

The enzymatic activity of MCM was detected using a modified protocol with ultra-performance liquid chromatography (UPLC) for measuring the production of succinyl-CoA from methylmalonyl-CoA.[10,17] Briefly, frozen cell pellets were re-suspended in 800 μL of 50 mmol/L sodium phosphate buffer, and then were lysed by sonication. The cell lysates were centrifuged and the resulting supernatant was used for the assay of MCM activity. An equal amount of the protein supernatant (90 μL) with 50 μL of 400 mmol/L sodium phosphate buffer and 10 μL of 180 μmol/L AdoCbl (Sigma-Aldrich, St Louis, MO, USA) was incubated at 37°C for 5 minutes. Reaction was initiated by adding 50 μL of 1.6 mmol/L methylmalonyl-CoA (Sigma-Aldrich, St Louis, MO, USA), and incubation was carried out at 37°C for 10 minutes. The reaction was terminated by the addition of 20 μL of 4.2 N $HClO_4$ (Sigma-Aldrich, St Louis, MO, USA) followed by centrifugation at 12 000 rpm for 10 minutes at 4°C. The resulting supernatant (170 μL) was neutralized by adding 30 μL of 1 mol/L Na_2CO_3, and then the reaction mixture of each sample was used for UPLC analysis. The reaction mixture without AdoCbl was used as a negative control. Methylmalonyl-CoA and succinyl-CoA were separated at 35°C by an UPLC system using an ACQUITY UPLC BEH C18 column (2.1×50 mm, 1.7 μm) with a mobile phase consisting of 85% solvent A [220 mmol/L phosphate

Table 1. Sequences of the primers used for site-directed mutagenesis PCR procedure

Missense mutations		Primer sequence (5'→3')	
c.419T>C (p.L140P)	CTG→C**c**G	Forward	ATCAGTTGCCTTTGATC**c**GGCGACACATC
		Reverse	**g**GATCAAAGGCAACTGATAATCCCTGCTGAC
c.421G>A (p.A141T)	GCG→**a**CG	Forward	CAGTTGCCTTTGATCTG**a**CGACACATCGTG
		Reverse	**t**CAGATCAAAGGCAACTGATAATCCCTGCTGAC
c.482G>T (p.G161V)	GGA→G**t**A	Forward	GATGTTGGAATGGCTG**t**AGTTGCTATTGACAC
		Reverse	**a**CAGCCATTCCAACATCACCACGAACTC
c.925T>G (p.W309G)	TGG→**g**GG	Forward	CAAGGTTGTCTTTCTTC**g**GGGGAATTGGAATG
		Reverse	**c**GAAGAAAGACAACCTTGGTGCAAATTCATC
c.1514T>C (p.I505T)	ATT→A**c**T	Forward	CGCTGTAGAAGTTCTGGCAA**c**TGATAATACTTCAG
		Reverse	**g**TTGCCAGAACTTCTACAGCGTCTTCTACAGCG
c.1540C>A (p.Q514K)	CAG→**a**AG	Forward	CTTCAGTGCGAAACAGG**a**AGATTGAAAAAC
		Reverse	**t**CCTGTTTCGCACTGAAGTATTATCAATTGCC
c.1790T>G (p.I597R)	ATA→A**g**A	Forward	GGAGAAAGTAAAGAGA**g**AACATCTGCTATC
		Reverse	**c**TCTCTTTACTTTCTCCAAATTCCTGGC
c.2168G>A (p.G723D)	GGT→G**a**T	Forward	GTTTCCAATGTATTTG**a**TCCTGGGACTCG
		Reverse	**t**CAAATACATTGGAAACACCAACTTCAAACAG

Lower-case bold letters represent the mutant bases in the primer sequences.

buffer (pH=4.0) with 14% (v/v) high performance liquid chromatography (HPLC) gradient grade methanol and 0.4% (v/v) HPLC gradient grade dichloromethane (Merck & Co., Inc., Whitehouse Station, NJ, USA)] and 15% solvent B (Milli-Q water) at a flow rate of 0.4-0.5 mL/min. Each of them was detected at 254 nm by a TUV detector. A stock solution of 5.5 mmol/L succinyl-CoA (Sigma-Aldrich, St Louis, MO, USA) was diluted into different concentrations and used for standard curve construction. Enzyme activity was expressed as nmol/min per mg protein.

Statistical analysis

Data were expressed as mean±standard error. Statistical analysis was performed using the GraphPad Prism software (GraphPad Software, Inc.). Unpaired *t* test was conducted with statistical significance established at two-tailed *P* value <0.05.

Results

Clinical characteristics of the patients

The clinical characteristics of the eight patients are shown in Table 2. The patients experienced their first metabolic crisis at age varying from 1 day to 8.8 years. They showed high levels of blood propionylcarnitine (C3) and urinary methylmalonyl acid. By the end of the study, 2 patients were lost to follow-up, 3 died, and 3 survived with mental retardation.

Predictive analysis of the effects of missense mutations on protein structure and function

The electrophoregrams of the eight *MUT* mutations are shown in Fig. 1. Bioinformatic analyses indicated that the eight missense mutations might have a high probability of affecting the MCM protein structure and function, and that possibly they are disease-causative (Table 3, Fig. 2).

Confirmation of wild-type and mutant plasmids

The presence of the recombinant wild-type plasmids carrying *MUT* cDNA was confirmed by agarose gel electrophoresis (Fig. 1B), and their sequences were verified (Fig. 1C). The mutant plasmids carrying the eight *MUT* missense mutations were verified respectively using sequencing and alignment (Fig. 1D). The results suggested that the wild-type and eight mutated expression vectors were successfully constructed.

Effect of mutations on MCM protein expression

MCM protein expression was determined by western blot. Significant differences were observed between the mutant plasmids G161V, W309G, Q514K, and G723D and the wild-type plasmid (P<0.01), as well as between the mutant plasmids L140P, A141T, and I505T and the wild-type plasmid (P<0.05) (Fig. 2). MCM protein

Table 2. Clinical characteristics of the eight patients

#	Gender	Age at onset	Clinical manifestations	Routine laboratory examinations	Blood C3 (normal: 0.5-4) (μmol/L)	Blood C3/C2 (normal: >0.25)	Urinary methylmalonic acid (normal: 0.2-3.6 mmol/mol Cr)	Prognosis§	*MUT* genotype‖
P1*	M	1 d	Poor feeding, failure to thrive, vomiting, lethargy, hypotonia, hepatomegaly	Metabolic ketoacidosis, anemia, abnormal myocardial enzymes	9.02	2.89	593.58	Death	**c.[925T>G];** [1630_1631delGGinsTA]
P2†	F	9 mon	Feeding difficulties, vomiting, lethargy, dyspnea, seizure, hypotonia	Metabolic acidosis, hyperammonemia, hyperlactacidemia, anemia	11.21	1.15	730.18	Death	**c.[323G>A];** **[1514T>C]**
P3‡	M	3 d	Poor feeding, failure to thrive, vomiting, lethargy, dyspnea, seizure	Metabolic acidosis, hyperammonemia, liver dysfunction	14.12	0.71	2902.82	Death	**c.[482G>T];** **[482G>T]**
P4	M	7 mon	Poor feeding, failure to thrive, vomiting, lethargy, respiratory abnormality, seizure, developmental delay, hypotonia	Hyperammonemia, hyperlactacidemia	13.57	0.47	286.85	Lost to follow up	**c.[419T>C];** [424A>G]
P5	M	4 d	Poor feeding, failure to thrive, vomiting, lethargy, polypnea, seizure, hypotonia, hepatomegaly	Metabolic acidosis, hyperammonemia, hyperlactacidemia, liver dysfunction, abnormal myocardial enzymes	32.82	0.70	632.08	3.5 y, mental retardation	c.[322C>T]; [323G>A; **2168G>A**]¶
P6	M	2.4 y	Vomiting, lethargy, dyspnea, developmental delay, hypotonia	Metabolic acidosis, liver dysfunction, abnormal renal function	8.01	1.13	2107.54	Lost to follow up	**c.[421G>A];** [810_811dupGG]
P7	M	22 d	Poor feeding, failure to thrive, vomiting, respiratory abnormality, lethargy, developmental delay, hypotonia, hepatomegaly	Metabolic acidosis, hyperammonemia, anemia, neutropenia, liver dysfunction, abnormal renal function, electrolyte disorder	36.87	1.40	747.07	3.5 y, mental retardation	c.[729_730insTT]; **[1540C>A]**
P8	M	8.8 y	Failure to thrive, vomiting, coma, seizure	No data	7.45	0.85	51.90	12 y, mental retardation	**c.[1790T>G];** [2106delA]

*: This patient had a family history; †: This patient was of the vitamin B12-responsive; ‡: This patient was diagnosed during newborn screening, and had been attacked when he was recalled; §: Follow-up ended in March 2015; ‖: The studied missense mutations are in bold (electropherograms are shown in Fig. 1); ¶: Two mutations were detected in paternal allele. M: male; F: female.

Table 3. Prediction of the effects of missense mutations on the methylmalonyl-CoA mutase (MCM) protein

Exon	Nucleotide change	Deduced amino acid change	Secondary structure that might be affected*	Protein functional domain that might be affected	PolyPhen-2 HumVar (score)	SIFT (score)
3	c.419T>C	p.L140P	α-helix	Substrate-binding region	Probably damaging (1.000)	Affect protein function (0.00)
3	c.421G>A	p.A141T	α-helix	Substrate-binding region	Probably damaging (0.989)	Affect protein function (0.00)
3	c.482G>T	p.G161V	β-turn	Substrate-binding region	Probably damaging (1.000)	Affect protein function (0.00)
5	c.925T>G	p.W309G	β-sheet	Substrate-binding region	Probably damaging (1.000)	Affect protein function (0.00)
8	c.1514T>C	p.I505T	-	Linker region	Probably damaging (0.993)	Affect protein function (0.00)
8	c.1540C>A	p.Q514K	α-helix	Linker region	Probably damaging (0.999)	Affect protein function (0.00)
10	c.1790T>G	p.I597R	α-helix	Cobalamin-binding region	Probably damaging (0.727)	Affect protein function (0.02)
13	c.2168G>A	p.G723D	β-turn	Cobalamin-binding region	Probably damaging (1.000)	Affect protein function (0.00)

*: The secondary structure was predicted using the human MCM crystal model (PDB:2XIJ). "-": none.

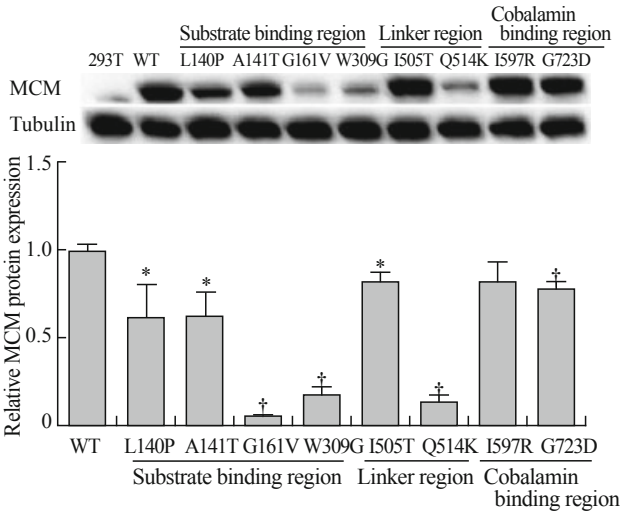

Fig. 2. Effect of the eight *MUT* mutations on methylmalonyl-CoA mutase (MCM) protein expression in HEK293T cells. The relative protein expression was normalized to HEK293T cells transfected with the wild-type plasmid. The data collected from three independent experiments were subjected to statistical analysis (*: $P<0.05$; †: $P<0.01$ *vs.* WT). WT: wild type.

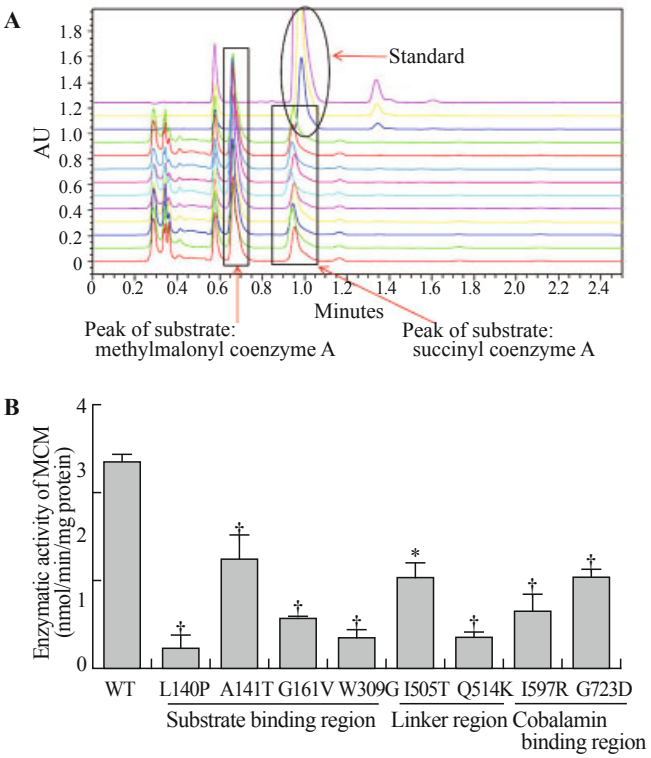

Fig. 3. Effect of the eight *MUT* mutations on methylmalonyl-CoA mutase (MCM) activity in HEK293T cells. **A**: A chromatogram illustrating the separation of methylmalonyl-CoA and succinyl-CoA by ultra-performance liquid chromatography (UPLC); **B**: The MCM activity of HEK293T cells transfected with wild-type and mutated plasmids (*: $P<0.05$; †: $P<0.01$ *vs.* WT). WT: wild type.

expression of the plasmid carrying I597R exhibited no significant difference ($P>0.05$). We confirmed that the Myc tag did not affect MCM expression.

Effect of the mutations on MCM activity

UPLC was used to evaluate the MCM activity by separating methylmalonyl-CoA and succinyl-CoA and then detecting the latter (Fig. 3A). Compared to the wild-type MCM activity, all eight mutants manifested a significantly decreased enzyme activity (all $P<0.05$) (Fig. 3B). Compared to the wild-type value, those of the residual enzyme activities were 9.9% for L140P, 53.3% for A141T, 24.4% for G161V, 6.2% for W309G, 43.9% for I505T, 14.9% for Q514K, 27.5% for I597R , and 44.1% for G723D.

Discussion

Missense mutations have indefinite effects on the protein as they do not necessarily cause significant changes in protein stability and activity.[13,18] In the present study, I597R did not affect the protein expression but its activity, whereas other mutations affected both protein expression and activity. We concluded that the eight missense mutations were loss-of-function mutations.

HEK293T cells also expressed in *MUT*, thus empty vector was used as a blank control. The measurement of the blank control was subtracted from the measurements of all plasmids to minimize the effect of intrinsic *MUT* expression in these cells. In addition, the experiments were repeated at least for three times, and the results

were consistent. Despite a C-terminal tagged allele was used and the activity was subsequently assayed under conditions that might prohibit the detection of mutations in the cobalamin-binding region, our results could indeed reflect the pathogenicity of the eight *MUT* mutations. Taken together, these results must be viewed as preliminary and they need further validation but support the causation. In the future, biochemical studies should be conducted on a cell line that lacks MCM activity and purify the C-terminal tagged allele to assay the Km and Vmax of mutant MCM for AdoCbl.

Further, we attempt to find the correlation of the genotype with the phenotype of the patients. Mutation G161V was identified at a homozygous state in patient 3 (P3), and the mutant protein expression and activity were decreased *in vitro*. Others[14,19] reported that mutations G161* and G161R at this position were speculated to be mut^0. Because the condition of the patient was severe three days after birth, we assumed that the mutation might be associated with the mut^0 phenotype. Patient 1(P1) with severe clinical manifestations one day after birth was found to carry compound heterozygous mutations W309G and G544*. G544* is a common mutation reported in the Chinese population, and homozygous patients can be identified to have mut^0.[14] W309G mutation occurs in the substrate-binding region, where most of the reported missense mutations are responsible for the mut^0 phenotype.[20] This mutant exhibited substantially decreased protein expression, and the protein activity was only 6.2% of that of the wild-type measured *in vitro* in this study, suggesting that W309G mutation might be designated mut^0.

In conclusion, the eight mutations (L140P, A141T, G161V, W309G, I505T, Q514K, I597R and G723D) in *MUT* assessed in this study displayed pathogenicity with loss of function. Further investigation is needed to better determine the residual enzyme activities with these novel missense mutations. The successfully constructed wild-type plasmid could be used for extensive investigation of the novel *MUT* mutations.

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