

4.2. Immunocytochemistry

Indirect immunocytochemistry was carried out either immediately after plating (for neurosphere staining) or after 4 DIV (for triple-labeling and for neuronal counting). The cells plated on the precoated chamber slides were fixed in 4% paraformaldehyde for 30 min, followed by permeabilization with 0.3% Triton X-100 for 5 min, and stained by immunofluorescence with the following primary antibodies: mouse anti-tubulin β III (1:200; Chemicon, Temecula, CA), mouse anti-MAP2 (1:500; Chemicon), mouse anti-nestin (1:200; Chemicon), rabbit anti-GFAP (1:400; Dako, Carpinteria, CA), and mouse anti-O4 (1:20, Chemicon). Primary antibodies were visualized with Cy3- (red), AMCA- (blue), and FITC- (green) conjugated secondary antibodies (Jackson Immuno-Research, West Grove, PA). 4',6-Diamidino-2-phenylindole (DAPI) was used as a fluorescent nuclear counterstain. Stained cultures were examined and photographed by fluorescence microscopy (Leica, Nussloch, Germany).

4.3. Statistical analysis

Statistical analyses were carried out using ANOVA or Student's *t* test. A *P* value <0.05 was considered significant.

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Mutation and haplotype analyses of the *MUT* gene in Japanese patients with methylmalonic acidemia

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Abstract Methylmalonic acidemia (MMA) is caused by a deficiency in the activity of L-methylmalonyl-CoA mutase (MCM), a vitamin B12 (or cobalamin, Cbl)-dependent enzyme. Apoenzyme-deficient MMA (*mut* MMA) results from mutations in the nuclear gene *MUT*. Most of the *MUT* mutations are thought to be private or restricted to only a few pedigrees. Our group elucidated the spectrum of mutations of Japanese *mut* MMA patients by performing mutation and haplotype analyses in 29 patients with *mut* MMA. A sequence analysis identified mutations in 95% (55/58) of the disease alleles. Five mutations were relatively frequent (p.E117X, c.385 + 5G > A, p.R369H, p.L494X, and p.R727X) and four were novel (p.M1V, c.753_753 + 5delGGTATA, c.1560G > C, and c.2098_2099delAT). Haplotype analysis suggested that all of the frequent mutations, with the exception of p.R369H, were spread by the founder effect. Among 46 Japanese patients investigated in the present and previous studies, 76% (70/92) of the mutations were located in exons 2, 6, 8, and 13. This finding – that a limited number of mutations account

for most of the mutations in Japanese *mut* MMA patients – is in contrast with results of a previous study in Caucasian patients.

Keywords Methylmalonic acidemia ·
L-Methylmalonyl-CoA mutase

Introduction

Methylmalonic acidemia (MMA) is an autosomal-recessive disorder of propionate metabolism caused by a defect in the isomerization of L-methylmalonyl-CoA to succinyl-CoA. The reaction is catalyzed by L-methylmalonyl-CoA mutase (MCM, EC 5.4.99.2), an enzyme which requires adenosylcobalamin (AdoCbl) as a cofactor (Fenton et al. 2001). MMA is classified into two forms: one resulting from a defect in the MCM apoenzyme (*mut* MMA or vitamin B₁₂-unresponsive MMA; MIM 251000) and another resulting from a defect in the steps leading to AdoCbl synthesis (*cbl* MMA or vitamin B₁₂-responsive MMA) (Rosenblatt and Fenton 2001). Typical MMA is characterized clinically by lethargy, vomiting, and hypertonia with abnormal movements, and biochemically by an accumulation of methylmalonic acid in the tissues and body fluid associated with hyperammonemia and ketoacidosis.

MCM is encoded by a single gene, *MUT*, which has been located to 6p21. *MUT* consists of 13 exons spanning 35 kb and it produces a 2.7-kb mRNA. To date, more than 100 disease-causing mutations in the human *MUT* gene have been reported (Ledley and Rosenblatt 1997; Acquaviva et al. 2005; Martinez et al. 2005), most of which seem to be unique or restricted to

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only a few pedigrees. However, there have been reports of specific mutations among various ethnic groups, including p.G717V in blacks (Adjalla et al. 1998), p.N219Y in Caucasians (Acquaviva et al. 2001), and p.R108C in Hispanics (Worgan et al. 2006). Ogasawara et al. (1994b) reported a relatively high incidence of p.E117X in Japanese patients and, more recently, Kobayashi et al. (2006) identified the plural occurrence of each of six mutations (p.L494X, p.R93H, p.E117X, p.R369H, p.G648D, and c.385 + 5G > A) in another Japanese population.

In study reported here, we performed mutation and haplotype analyses on 29 Japanese patients with *mut* MMA to examine the spectrum of mutations within the population and explore the possibility of a molecular diagnosis.

Methods

Patients

Twenty-nine apparently unrelated *mut* MMA patients were studied in the present investigation. There were no consanguineous marriages among the parents of these patients. Patient 6 was a Brazilian descended from Japan immigrants. All of the patients with available clinical information had been symptomatic during their neonatal or infantile periods. Responsiveness to vitamin B12 was not found in all of the patients except patient 7. The oral administration of vitamin B12 in patient 7 reduced the urinary excretion of methylmalonic acid clinically, and the fibroblasts from this patient showed an increased incorporation of ^{14}C -propionate – from 7 to 40%

– following the administration of vitamin B12 in vitro (case 2 in Kakinuma et al. 1985). This patient was classified as *cbl* MMA on the basis of these findings, but neither the *MMAA* nor *MMAB* mutation was found (Yang et al. 2004). Patient 23 exhibited one of the mutations (p.V669E), but another mutation was not found in the previous study (cell No. 69 in Mikami et al. 1999). The diagnoses of MMA were confirmed by urinary organic acid analysis using gas chromatography and mass spectrometry. The MCM assay was performed in most cases by measuring the isomerization of L-methylmalonyl-CoA to succinyl-CoA by means of high-performance liquid chromatography (Kikuchi et al. 1989), and MCM activity was undetectable in all of those assayed. We could not successfully subclass the patients into *mut*⁰ or *mut*⁻ by this method.

Direct sequencing of the *MUT* genes

Genomic DNA was extracted from cultured fibroblasts, EBV-transformed lymphoblasts, or leukocytes with the aid of a Sepa Gene Kit (Sanko Junyaku, Tokyo, Japan). All coding exons, including flanking introns in *MUT*, were amplified by PCR (Table 1). To facilitate the cycle sequencing analysis, the KS primer sequence and M13 reverse primer sequence were attached to the 5' ends of the sense primers and antisense primers, respectively. The PCR products were directly sequenced using a Big Dye Primer Cycle Sequencing kit and an ABI 310 Genetic Analyzer (PE Applied Biosystems, Foster City, Calif.).

The Ethics Committee of the Tohoku University School of Medicine approved this study.

Table 1 Primers for the amplification of the *MUT* gene

Sense primer ^a		Antisense primer ^b	
MUT-Ex2-KS	KS-GAGTAGCTCCTATTTCCCAC	MUT-Ex2-Rev	Rev-GAGTGAATATCATCTTTACA
MUT-Ex3-KS	KS-ACCTTGATTCCAGACTCTTG	MUT-Ex3-Rev	Rev-CTACATTCAAGGAACATATAG
MUT-Ex4-KS	KS-AGTCCTGATGATGGTTTCATG	MUT-Ex4-Rev	Rev-ATCTAAATCTAGCCTGACAT
MUT-Ex5-KS	KS-TGTACGTGCACTGATCTTAA	MUT-Ex5-Rev	Rev-CTTGTGCCACATTGCTCAGA
MUT-Ex6-KS	KS-GCTATTCTGAAGCTTAATAT	MUT-Ex6-Rev	Rev-TATAAATCTTGACTTGTAAG
MUT-Ex7-KS	KS-TGATGTTTATTTAATTCTGT	MUT-Ex7-Rev	Rev-GTGCATCCATGTATGTGAAA
MUT-Ex8-KS	KS-CTCAGATTGGGATTGCTGA	MUT-Ex8-Rev	Rev-CACCTCATGCTGTTGTAAGG
MUT-Ex9-KS	KS-ATGCTATGCATCAGGGTCTA	MUT-Ex9-Rev	Rev-ACATGGTTTACAGGATCAAC
MUT-Ex10-KS	KS-GAATTGGATGCATAAAGGCA	MUT-Ex10-Rev	Rev-TTCTCAGTTGTATGTAAGG
MUT-Ex11-KS	KS-CTTGAAAGATTGCTGTGAA	MUT-Ex11-Rev	Rev-TACCAGTTACCAGGAGATGT
MUT-Ex12-KS	KS-GCCCATAGTATGTTCTGAA	MUT-Ex12-Rev	Rev-ACACTGTCCACTTTTAGACC
MUT-Ex13-KS	KS-TGCCAGTAGTATACCAGTTG	MUT-Ex13-Rev	Rev-GAAGACATAGCTTTACTCTC

^a KS, CGAGGTCGACGGTATCG

^b Rev, CAGGAAACAGCTATGAC

Table 2 Mutations and single nucleotide polymorphisms in the *MUT* gene

Patient number	Gender	Onset	Allele 1	Allele 2	Single nucleotide polymorphisms									
					c.636	c.1084	c.1495	c.1560	c.1676	c.1677	c.1677	c.1677	c.1808	c.2011
					A > G	A > G	G > A	G > A	A > C	A > T	A > G	A > G	C > G	G > A
					rs2229384	rs17851388	rs9473558	rs9381786	rs9463483	rs9473557	rs9473555	rs6458687		
Pt1	F	7 days	c.322C > T (p.R108C)	c.2098_2099 delAT (p.M700 V fsX10)	G A	A A	G G	ND	A A	ND	A G	C G	A G	A G
Pt2	M	5 days	c.349G > T (p.E117X)	c.349G > T (p.E117X)	G G	G G	G G	16 16	A A	A A	A A	C C	A A	A A
Pt3	M	- ^a	c.349G > T (p.E117X)	c.148IT > A (p.L494X)	G G	G G	G G	16 16	A A	A A	A A	C C	A A	A A
Pt4	M	Neonate	c.349G > T (p.E117X)	c.2179C > T (p.R727X)	G A	G A	G G	16 17	A A	A T	A G	C G	A G	A G
Pt5	M	28 days	c.385+5G > A (IVS2+5G > A)	c.385+5G > A (IVS2+5G > A)	G G	A A	G G	ND	A A	A A	A A	C C	A A	A A
Pt6	M	4 days	c.385+5G > A (IVS2+5G > A)	c.385+5G > A (IVS2+5G > A)	G G	ND	ND	ND	ND	ND	ND	ND	ND	A A
Pt7	F	8 months	c.385+5G > A (IVS2+5G > A)	c.1106G > A (p.R369H)	G G	A A	G G	ND	A A	ND	A A	C C	A A	A A
Pt8	M	23 days	c.385+5G > A (IVS2+5G > A)	c.1106G > A (p.R369H)	G A	A A	G G	16 17	A A	A T	A G	C G	A G	A G
Pt9	M	3 days	c.385+5G > A (IVS2+5G > A)	c.1106G > A (p.R369H)	G A	A A	G G	16 17	A A	A T	A G	C G	A G	A G
Pt10	F	- ^a	c.385+5G > A (IVS2+5G > A)	c.148IT > A (p.L494X)	G G	A G	G G	ND	A A	A A	A A	C C	A A	A A
Pt11	M	2 days	c.385+5G > A (IVS2+5G > A)	c.1560G > C (p.K520K, splice error?)	G A	A A	G G	16 17	A A	A T	A G	C G	A G	A G
Pt12	M	1 day	c.753_753 +5del6bp (splice error?)	c.753_753 +5del6bp (splice error?)	G G	A A	G G	16 16	A A	A A	A A	C C	A A	A A
Pt13	F	9 days	c.323G > A (p.R108H)	c.1105C > T (p.R369C)	G A	A A	G G	16 16	A C	A A	A A	C C	A G	A G
Pt14	F	11 months	c.1106G > A (p.R369H)	c.1A > G (p.M1V)	G A	A A	G G	16 16	A C	A A	A A	C C	A G	A G
Pt15	F	10 months	c.1106G > A (p.R369H)	c.1105C > T (p.R369C)	A A	A A	G A	17 16	A C	T A	G A	G C	G G	G G

Table 2 continued

Patient number	Gender	Onset	Allele 1	Allele 2	Single nucleotide polymorphisms									
					c.636	c.1084	c.1495	c.1560	c.1595	c.1676	c.1677	c.1677	c.1808	c.2011
Pt16	F	– ^a	c.1106G > A (p.R369H)	Nil found	A > G	A > G	G > G	17 16	A G	A A	T A	G A	G C	G A
Pt17	M	– ^a	c.1481T > A (p.L494X)	c.1481T > A (p.L494X)	G G	G G	G G	16 16	G G	A A	A A	A A	C C	A A
Pt18	M	– ^a	c.1481T > A (p.L494X)	c.1481T > A (p.L494X)	G G	G G	G G	16 16	G G	A A	A A	A A	C C	A A
Pt19	M	– ^a	c.1481T > A (p.L494X)	c.1105C > T (p.R369C)	G A	G A	G G	16 16	G G	A C	A A	A A	C C	A G
Pt20	M	14 days	c.1481T > A (p.L494X)	c.1560+1G > T (IVS8+1G > T)	G A	G A	G G	16 17	G A	A A	A T	A G	C G	A G
Pt21	F	3 days	c.1481T > A (p.L494X)	Nil found	G A	G A	G G	16 17	G A	A A	A T	A G	G C	A G
Pt22	M	– ^a	c.1481T > A (p.L494X)	Nil found	G A	G A	G G	16 16	G G	A C	A A	A A	C C	A G
Pt23	F	– ^a	c.1560+1G > T (IVS8+1G > T)	c.2006T > A (p.V669E)	A A	A A	G G	17 17	A A	A A	T T	G G	G G	G G
Pt24	F	6 months	c.1560+1G > T (IVS8+1G > T)	c.2179C > T (p.R727X)	A A	A A	G G	17 17	A A	A A	T T	G G	G G	G G
Pt25	M	9 months	c.1560+1G > T (IVS8+1G > T)	c.2179C > T (p.R727X)	A A	A A	G G	ND	A A	A A	T T	G G	G G	G G
Pt26	F	Neonate	c.1962delT (p.P654fsX17)	c.1962delT (p.P654fsX17)	G G	A A	G G	16 16	G G	A A	A A	A A	C C	A A
Pt27	M	4 months	c.2179C > T (p.R727X)	c.2179C > T (p.R727X)	A A	A A	G G	17 17	A A	A A	T T	G G	G G	G G
Pt28	F	7 months	c.2179C > T (p.R727X)	c.689_690delCA (p.T2311fsX13)	A A	A A	G A	17 16	A G	A C	T A	G A	G C	G G
Pt29	M	11 months	c.2179C > T (p.R727X)	c.2080C > T (p.R694W)	A G	A A	G G	17 16	A G	A A	T A	G A	G C	G A

ND not done

^a Clinical information was not available

Results and discussion

Twenty-nine *mut* MMA patients were studied for mutation analysis. Sequence analysis identified mutations in 95% (55/58) of the disease alleles (Table 2), with 17 mutations being identified in total. Three of the patients (16, 21, and 22) had only one mutation as a heterozygous change each.

Four mutations were novel (p.M1V, c.753_753 + 5delGGTATA, c.1560G > C, and c.2098_2099delAT). The mutation in the translation initiation codon, M1V, has been reported to be pathogenic in other diseases (Lyonnet et al. 1992; Cheadle et al. 1994). The presence of a splice donor site in intron 3 of c.753_753 + 5delGGTATA suggests that this deletion plays a pathogenic role. The sequence flanking this deletion exhibits an intrastrand complementarity (CAAAGGTATACTTTG). It is hypothesized that c.753_753 + 5delGGTATA is associated with the formation of the hairpin loop structure in a single-strand DNA (Robinson et al. 1997). A c.1560G > C substitution was identified at the 3' end of exon 8 that

appeared to result in missense-mediated splicing errors. A two-base deletion, c.2098_2099delAT, resulted in a frame-shift and a premature termination.

The total allelic frequency of four mutations (c.385 + 5G > A, p.R369H, p.L494X, and p.R727X) was 55% (32/58). The p.E117X mutation, previously reported as a relatively frequent mutation in Japanese, was found in four alleles (7%) in this study. The mutations p.R93H and p.G648D each appeared more than once in the data from Kobayashi et al. (2006), whereas our data revealed neither. The mutations p.G717V (common mutation in black) and p.N219Y (common mutation in Caucasians) were not found, and p.R108C (common mutation in Hispanic) was detected in only one allele in this study. Figure 1 summarizes the mutations found in 46 Japanese patients (Ogasawara et al. 1994a, b; Toyo-Oka et al. 1995; Mikami et al. 1999; Kobayashi et al. 2006). Worgen et al. (2006) identified exons 2, 3, 6, and 11 as mutation clusters, whereas our data on Japanese patients indicated that 76% (70/92) of the mutations were located in exons (and flanking introns) 2, 6, 8, and 13.

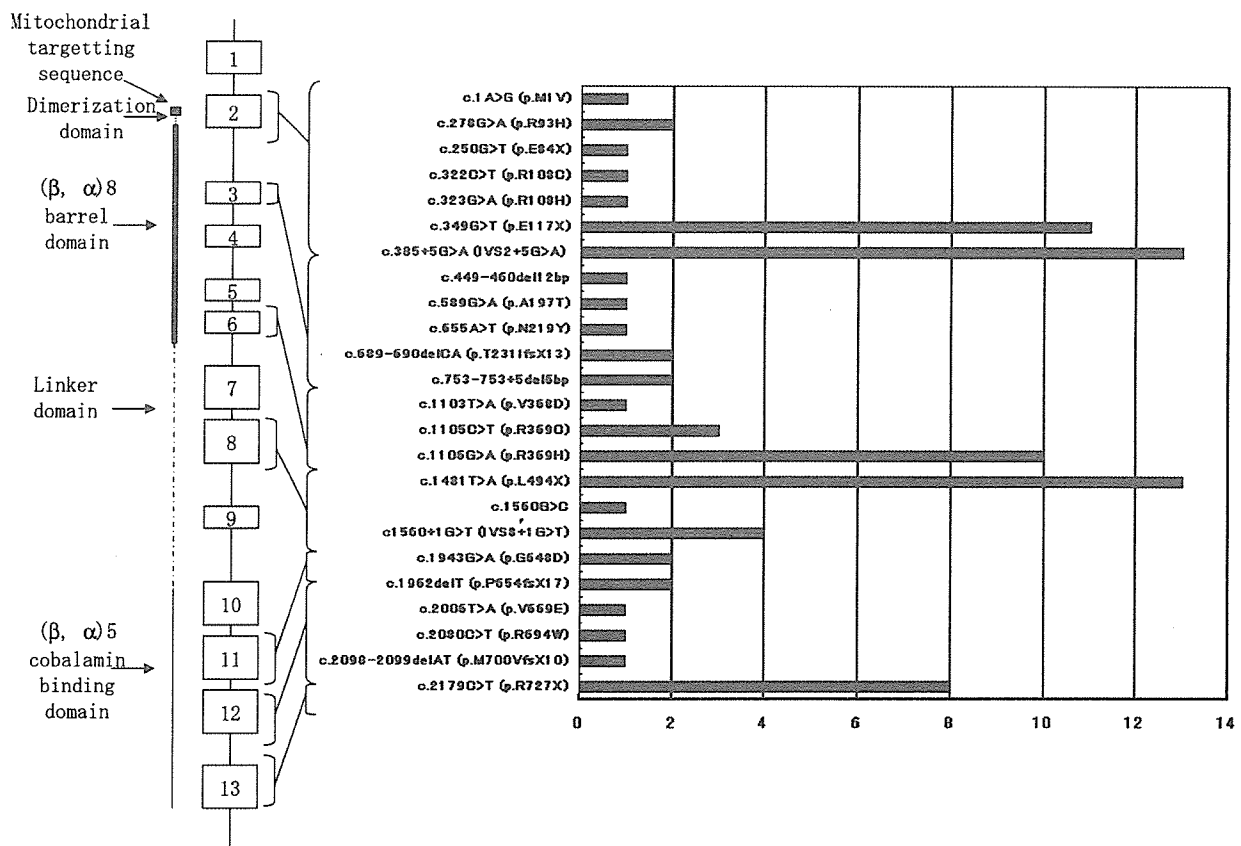


Fig. 1 The distribution of mutations found in 46 Japanese *mut* MMA patients (Ogasawara et al. 1994a, b; Toyo-Oka et al. 1995; Mikami et al. 1999; Kobayashi et al. 2006; and this study)

Table 3 Haplotypes and linked mutations

	c.636 A > G rs2229384	c.1084-50 A > G	c.1495 G > A rs17851388	c.1560+16 Tn	c.1595 G > A rs94735558	c.1676+77 A > C rs9381786	c.1677-164 A > T rs9463483	c.1677-53 A > G rs9473557	c.1808+66 C > G rs9473555	c.2011 G > A rs6458687
Haplotype 1 c.349G > T (p.E117X) c.1481T > A (p.L494X)	G	G	G	16	G	A	A	A	C	A
Haplotype 2 c.323G > A (p.R108H) c.385+5G > A (IVS2+5G > A) c.753_753+5del6bp c.1106G > A (p.R369H) c.1962delT (p.P654fsX17) c.2080C > T (p.R694W)	G	A	G	16	G	A	A	A	C	A
Haplotype 3 c.1A > G (p.M1V) c.1105C > T (p.R369C)	A	A	G	16	G	C	A	A	C	G
Haplotype 4 c.689_690delCA (p.T231IfsX13) c.1105C > T (p.R369C)	A	A	A	16	G	C	A	A	C	G
Haplotype 5 c.1106G > A (p.R369H) c1560G > C c1560+1G > T (IVS8+1G > T) c.2006T > A (p.V669E) c.2179C > T (p.R727X)	A	A	G	17	A	A	T	G	G	G
Allele frequency among Japanese (by HapMap Project ^a)	A:0.409 G:0.591				G:0.830 A:0.170	A:0.844 C:0.156			C:0.826 G:0.174	G:0.318 A:0.682

^a The International HapMap Consortium (2003), <http://www.hapmap.org/>

With respect to genotype-phenotype correlations, patient 2 was homozygous for the p.E117X and manifested symptoms on the fifth day of life. Ogasawara et al. (1994b) reported a patient homozygous for p.E117X who showed initial symptoms at the age of 9 months. Patients 5 and 6 were homozygous for c.385 + 5G > A, and the onset was neonatal in both cases: patient 5 is still alive (now 7 years old) and patient 6 died after the first attack. Three patients (7, 8, and 9) were compound heterozygotes of c.385 + 5G > A and p.R369H: patient 7 manifested symptoms at 8 months and these have been kept under well control with vitamin B12 treatment; patients 8 and 9 both showed initial symptoms in the neonatal period and did not respond to vitamin B12. In a previous kinetics study, the V_{\max} value of the p.R369H-mutant enzyme was only 1% of that of the wild type (Janata et al. 1997), and Toyo-Oka et al. (1995) reported that reverse transcription (RT)-PCR did not demonstrate the presence of a normally spliced transcript from fibroblasts of a homozygote of c.385 + 5G > A. We have no explanation why patient 7 responds to vitamin B12 treatment. The clinical features seem to correlate not only with genotype, but also with other unknown environmental factors, such as the nutritional state and/or modifier genes.

Ten single nucleotide polymorphisms (SNPs) were found in the sequenced region, and the haplotypes could be separated into five groups based on five SNP patterns (Table 3). Among the more frequent mutations, p.E117X and p.L494X were linked to haplotype 1; c.385 + 5G > A was linked to haplotype 2; p.R727X was linked to haplotype 5. These frequent mutations appeared to have been spread by the founder effect in the Japanese population. Mutation p.R369H, on the other hand, may be of a double origin (haplotypes 2 and 5). The p.R369 codon contains a CpG dinucleotide, and p.R369H has been found in Turkish, Greek, Caucasian, Hispanic (Worgan et al. 2006), and Korean (Jung et al. 2005) populations.

In conclusion, a limited number of mutations accounted for most of the Japanese *mut* MMA patients, which is in contrast to the results of a previous study on Caucasian patients. Hopefully the results reported here will facilitate the DNA diagnosis of *mut* MMA within the Japanese population.

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