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Clinical and molecular findings in Thai patients with isolated methylmalonic acidemia

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ABSTRACT

Isolated methylmalonic acidemia (MMA) is a genetically heterogeneous organic acid disorder caused by either deficiency of the enzyme methylmalonyl-CoA mutase (MCM), or a defect in the biosynthesis of its cofactor, adenosyl-cobalamin (AdoCbl). Herein, we report and review the genotypes and phenotypes of 14 Thai patients with isolated MMA. Between 1997 and 2011, we identified 6 *mut* patients, 2 *cblA* patients, and 6 *cblB* patients. The *mut* and *cblB* patients had relatively severe phenotypes compared to relatively mild phenotypes of the *cblA* patients. The *MUT* and *MMAB* genotypes were also correlated to the severity of the phenotypes. Three mutations in the *MUT* gene: c.788G>T (p.G263V), c.809_812dupGGGC (p.D272Gfs*2), and c.1426C>T (p.Q476*); one mutation in the *MMAA* gene: c.292A>G (p.R98G); and three mutations in the *MMAB* gene: c.682delG (p.A228Pfs*2), c.435delC (p.F145Lfs*69), and c.585-1G>A, have not been previously reported. RT-PCR analysis of a common intron 6 polymorphism (c.520-159C>T) of the *MMAB* gene revealed that it correlates to deep intronic exonization leading to premature termination of the open reading frame. This could decrease the ATP:cobalamin adenosyltransferase (ATR) activity resulting in abnormal phenotypes if found in a compound heterozygous state with a null mutation. We confirm the genotype–phenotype correlation of isolated MMA in the study population, and identified a new molecular basis of the *cblB* disorder.

1. Introduction

Isolated methylmalonic acidemia (MMA, OMIM 251000) is a genetically heterogeneous inborn error of metabolism characterized by abnormal accumulation of methylmalonyl-CoA and methylmalonic acid in body fluids without hyperhomocysteinemia. Isolated MMA can be caused by either a defect of the MCM methylmalonyl-CoA mutase (MCM; EC 5.4.99.2) or one of the defects in the synthesis of its cofactor adenosyl-cobalamin (AdoCbcl). Defects of MCM (OMIM 251000) can be subdivided into two subgroups: mut^- with residual activity in the presence of AdoCbl, and mut^0 with complete loss of MCM activity [1]. Defects of AdoCbl synthesis leading to isolated MMA can be categorized into three complementation groups: *cblA* (OMIM 607481), *cblB* (OMIM 607568), and *cblD*-variant 2 (OMIM 606169).

Patients with isolated MMA typically present with recurrent vomiting, respiratory distress, progressive alteration of consciousness, overwhelming illness, deep coma, and death [2]. The common laboratory features are severe ketoacidosis, hypo- or hyperglycemia, neutropenia/anemia/pancytopenia, and hyperammonemia. With appropriate therapy, most patients survive the acute metabolic crisis but still suffer from long-term complications, such as chronic renal

Abbreviations: AdoCbl, adenosyl-cobalamin; ATR, ATP:cobalamin adenosyltransferase; CKD, chronic kidney disease; ESE, exon splicing enhancer; GC–MS, gas chromatography–mass spectrometry; LVEF, left ventricular ejection fraction; MCM, methylmalonyl-CoA mutase; MMA, methylmalonic acidemia; NMD, nonsense-mediated decay; PTC, premature termination codon; RTA, renal tubular acidosis.

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failure, failure to thrive, and developmental delay [3–5]. The long-term outcome of patients with early-onset disease and cobalamin-unresponsive phenotype is unfavorable [6,7]. The severity and long-term outcome are also influenced by the underlying enzymatic defects [5,8]. Patients with the *mut*^{0} and *cblB* defects exhibit a more severe phenotype, and higher frequency of complications than those of *mut*$^-$ and *cblA* [5]. The four genes known to cause isolated MMA include *MUT*, *MMAA*, *MMAB*, and *MMADHC* genes which are responsible for the *MCM*, *cblA*, *cblB*, and *cblD*-variant2 defects, respectively [9–12]. The mutations identified in these genes are molecularly heterogeneous [13–17].

In this study, we report and review the clinical and biochemical features, and genetic defects in the *MUT*, *MMAA*, and *MMAB* genes of 14 Thai patients affected with isolated MMA. Clinical and biochemical (complementation group) phenotypes were correlated with the genotypes identified. We demonstrate that a common polymorphism residing in intron 6 of the *MMAB* gene is a potential highly pathogenic allele via enhancement of alternative splicing.

2. Patients and methods

2.1. Patients and MMA diagnosis

The study includes Thai patients with isolated MMA and their families, who were diagnosed and have been followed up at the Departments of Pediatrics of four medical institutes in Bangkok: Siriraj Hospital, Chulalongkorn Memorial Hospital, Phramongkutklao Hospital, and Ramathibodi Hospital between June, 1997 and June, 2011. All patients were diagnosed by urine organic acid analysis using urease treatment extraction [18] with gas chromatography–mass spectrometry (GC–MS) after presenting clinical symptoms, and not through neonatal screening. All patients exhibited increased urinary excretions of methylmalonate with or without 3-hydroxypropionate and methylcitrate. The previously reported genotypes and phenotypes of patients 1, 2, 3, 9, and 10 [19–21], as well as their additional clinical and molecular findings, were included in this study. In vivo responsiveness to oral methylcobalamin or cyanocobalamin (2–10 mg/day), or intramuscular hydroxycobalamin (1 mg/day) was defined as a decrease in urinary methylmalonate excretion of more than 50% or to an undetectable level after more than one-week administration of the cobalamin [22]. Metabolic treatments, including protein restriction (with administration of isoleucine-, methionine-, threonine-, and valine-free special formulas), oral carnitine supplementation, intermittent eradication of gut flora by metronidazole or neomycin, and cobalamin (either oral or intramuscular administration), were given to all patients. Emergency treatment was performed during acute metabolic crises. Informed consent was obtained from the families of the patients. The study was approved by the Institutional Ethical Review Board of Faculties of Medicine of Siriraj Hospital and Ramathibodi Hospital, Mahidol University.

2.2. Enzymatic and mutation analyses

MCM activity in lymphocytes was determined in vitro by measuring the isomerization of L-methylmalonyl-CoA to succinyl-CoA by high performance liquid chromatography, as previously described [23,24]. AdoCbl cofactor was included in the reactions. The patients with decreased or undetectable MCM activity were classified together as the *mut* complementation group.

The lymphocytes were also used as sources of DNA and RNA. Mutations of the *MUT*, *MMAA*, and *MMAB* genes from affected individuals and their parents were determined by direct sequencing of genomic DNA or cDNA, as previously described [20]. Sequencing of the *MUT* gene was only performed in the patients with defects in MCM activity, and sequencing of the *MMAA* and *MMAB* genes was performed in the patients with normal MCM activity. For cDNA analysis, total RNA was extracted with the QIAamp RNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany) and was converted to cDNA with Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and the oligo dT_{15} primer. Fifty Thai control individuals were screened for each mutation and polymorphism identified in this study. Mutation designation was according to the official mutation nomenclature (http://www.hgvs.org/mutnomen/). Nucleotide numbering is based on cDNA reference sequences GenBank accession numbers NM_000255, NM_172250, and NM_052845 for *MUT*, *MMAA*, and *MMAB*, respectively.

To examine the effects of the c.520-96, -128, and -159C>T polymorphisms identified in intron 6 of the *MMAB* gene in patient 9, the fragment of the *MMAB* cDNA from exon 5 to exon 9 (surrounding this intron) was PCR-amplified with the primers CCATACATTTGCCGAAGAGC and CAAGCTCCCACTTTCTGTGA, and then directly sequenced. The relative intensities of the bands amplified with these primers were also compared after agarose gel electrophoresis and ethidium bromide staining. A β-actin fragment was PCR-amplified with the primers AACACCCCAGCCATGTACG and ATGTCACGCACGATTTCCC, as a cDNA loading control. A bioimaging system (Syngene, England) was used to capture and analyze the PCR band intensity.

3. Results

3.1. Clinical features

A total of 14 patients representing 13 Thai families (patients 13 and 14 are siblings) are included in this study. The clinical features and complementation groups of all patients are summarized in Table 1. Nine out of 14 patients (64%) presented in the neonatal period (between the ages of 2 and 17 days), while the remaining five patients presented in the later infancy period (between the ages of 5 and 8 months). All late-onset patients had the first metabolic crisis triggered by infections. Consanguinity was reported in seven out of 13 families (54%). The parents of patient 4 are mountain tribe people, namely Lua. The interval from the age of onset to the age at diagnosis was between 2 days and 7 months, except for patient 14, who had an older sibling (patient 13) known to have MMA and was diagnosed 1 day after developing the symptoms at the age of 4 days. Three patients died during subsequent metabolic crises. Eleven patients are alive (ages range from 12 months to 14 years). Eight out of 11 patients (73%) older than two years of age had some degree of renal involvement. Patient 3 had early-onset and rapidly progressive renal complications, namely renal tubular acidosis (RTA) type 4 and chronic kidney disease (CKD) stage 3, despite good metabolic control. Patient 12 developed dilated cardiomyopathy with decreased left ventricular ejection fraction (LVEF) of 31% (normal > 55%) and basal ganglia infarction during a severe metabolic episode at 6 months of age. L-Carnitine was increased to 200 mg/kg/day, and furosemide, digoxin, and spironolactone were started. A repeat echocardiogram at 2 years of age showed some improvement of cardiomyopathy and borderline LVEF of 51%. Eight out of 14 patients (57%) had variable neurologic complications, including mental retardation (IQ < 70), delayed development, epilepsy, and extrapyramidal symptoms from basal ganglia infarction. Growth failure (either weight or height lower than 3rd percentile) was identified in six out of 14 cases (43%). Overall, nine out of 11 cases (82%) who were older than 2 years of age were affected with one of the long-term complications related to isolated MMA.

3.2. Enzymatic and mutation analyses

Of the 14 patients, 6 were classified as *mut*, 2 as *cblA*, and 6 as *cblB* disorders. Overall, we successfully identified 28 mutant alleles including 8, 2, and 6 different *MUT*, *MMAA*, and *MMAB* mutant alleles, respectively (Table 2). Polymorphisms in the *MUT* and *MMAB* genes were also identified (Table 3). Eight patients had homozygous mutations, which

Table 1
Clinical characterization of isolated MMA patients categorized by the complementation groups.

Patient	Complementation group	Current age	Age of onset	Age of diagnosis	Parental consanguinity	B12 response	Renal involvement (onset)	Current growth (percentile of wt./ht.)	Development	Other complications
1[a]	*Mut*	10 y	2 d	10 d	No	NS	CKD stage 2	50th/<3rd	Moderate MR (IQ = 47)	
2[b]	*Mut*	Died at 6 y	10 mo	10 mo	Yes	NS	None	3rd/3rd	Mild MR (IQ = 57)	Basal ganglia lesions
3[c]	*Mut*	6 y	5 mo	7 mo	No	Yes	RTA type 4 (2 y) CKD stage 3	50th/50th	Normal (DQ = 100)	
4	*Mut*	4 y	6 mo	8 mo	Yes	Yes	None	25th/25th	Normal (DQ = 97)	
5	*Mut*	3 y 7 mo	4 d	7 d	No	No	RTA type 2 (18 mo)	97th/25th	Mild delay (DQ = 69)	Recurrent pancreatitis
6	*Mut*	Died at 19 mo	4 d	5 mo	Yes	NS	RTA type 4 (16 mo)	3rd/3rd	Severe delay (DQ = 31)	Subdural hematoma at 3 m
7	*CblA*	5 y	7 mo	11 mo	Yes	Yes	None	25th/25th	Normal (DQ = 85)	
8	*CblA*	4 y 8 mo	8 mo	9 mo	Yes	Yes	RTA type 4 (3 y)	25th/50th	Normal (DQ = 100)	
9[b]	*CblB*	14 y	17 d	5 mo	No	NS	RTA type 1 (6 y)	3rd/<3rd	Moderate MR (IQ = 48)	
10[b]	*CblB*	12 y	6 d	7 mo	Yes	No	RTA type 1 (4 y), HT, CKD stage 3	<3rd/<3rd	Mild MR (IQ = 62)	
11	*CblB*	5 y	9 d	1 mo	Yes	Yes	RTA, CKD stage 2 (3 y)	10th/<3rd	Normal (IQ = 85)	
12	*CblB*	2 y	6 d	8 d	No	NS	RTA (12 mo)	10th/10th	Moderate delay (DQ = 44)	Cardiomyopathy at 6 mo, basal ganglia infarction
13	*CblB*	Died at 19 mo	3 d	1 mo	No	NS	None	50th/25th	Moderate delay	Epilepsy at 12 mo
14[d]	*CblB*	12 mo	3 d	4 d	No	NS	None	50th/25th	Normal	

Y, year; mo, month; d, day; wt., weight; ht., height; NS, not studied, RTA, renal tubular acidosis; HT, hypertension; MR, mental retardation; IQ, intelligence quotient; DQ, developmental quotient.
eGFR, estimated glomerular filtration rate or CrCl, creatinine clearance (normal > 90 ml/min/1.73 m^2); CKD, chronic kidney disease (stage 2 = GFR 60–89 ml/min/1.73 m^2, stage 3 = GFR 30–59 ml/min/1.73 m^2).
[a] The genotypes were published in Ref. [19].
[b] The genotypes were published in Ref. [20].
[c] The genotype was published in Ref. [18].
[d] A younger brother of patient 13.

are related to parental consanguinity. We identified three novel mutations in the *MUT* gene: c.788G>T (p.G263V), c.809_812dupGGGC (p.D272Gfs*2), and c.1426C>T (p.Q476*); one novel mutation in the *MMAA* gene: c.292A>G (p.R98G); and three novel mutations in the *MMAB* gene: c.682delG (p.A228Pfs*2), c.435delC (p.F145Lfs*69), and c.585-1G>A. The two novel missense mutations: p.G263V in the *MUT* gene, and p.R98G in the *MMAA* gene are likely to be pathogenic. To support this prediction, we compared the MCM and MMAA protein sequences between several species, and found that these 2 amino acid codons are 100% conserved across all species (data not shown). All novel *MCM*, *MMAA*, and *MMAB* mutations have not been reported in the locus specific databases including, the Human Gene Mutation Database (http://www.hgmd.org) and Leiden Variation Open Database (http://www.genomed.org/lovd/), as well as the National Center for Biotechnology (NCBI) SNP database (http://www.ncbi.nlm.nih.gov/SNP). They were not present in 100 alleles from Thai controls.

Table 2
Genotypes of isolated MMA patients categorized by the complementation groups.

Patient	Gene	MCM activity (pmol/min/mg)[a]	Exon/intron	Nucleotide change (first allele)	Predicted protein change (first allele)	Exon/intron	Nucleotide change (second allele)	Predicted protein change (second allele)
1[b]	*MUT*	ND	Ex. 5	c.972delT	p.A324Afs*9	Ex. 9	c.1630_1631delGGinsTA	p.G544*
2[c]	*MUT*	ND	Ex. 2	c.91C>T	p.R31*	Ex. 2	c.91C>T	p.R31*
3[d]	*MUT*	ND	IVS11	c.1957-2A>G	Affect splicing of ex. 12	IVS11	c.1957-2A>G	Affect splicing of ex. 12
4	*MUT*	ND	Ex. 12	c.2080C>T	p.R694W	Ex. 12	c.2080C>T	p.R694W
5	*MUT*	ND	Ex. 4	**c.788G>T**	**p.G263V**	Ex. 4	**c.809_812dupGGGC**	**p.D272Gfs*2**
6	*MUT*	ND	Ex. 7	**c.1426C>T**	**p.Q476***	Ex. 7	**c.1426C>T**	**p.Q476***
7	*MMAA*	644	Ex. 2	c.433C>T	p.R145*	Ex. 2	c.433C>T	p.R145*
8	*MMAA*	441	Ex. 2	**c.292A>G**	**p.R98G**	Ex. 2	**c.292A>G**	**p.R98G**
9[c]	*MMAB*	482	Ex. 6	c.454G>T	p.E152*	IVS6	c.520-96, -128, -159C>T	Deep intronic exonization
10[c]	*MMAB*	304	Ex. 6	c.454G>T	p.E152*	Ex. 6	c.454G>T	p.E152*
11	*MMAB*	704	Ex. 9	**c.682delG**	**p.A228Pfs*2**	Ex. 9	**c.682delG**	**p.A228Pfs*2**
12	*MMAB*	531	Ex. 6	**c.435delC**	**p.F145Lfs*69**	Ex. 7	c.563_577dup	p.V188_A192dup
13, 14	*MMAB*	400, NA	IVS7	**c.585-1G>A**	**Affect splicing of ex. 8**	Ex. 7	c.563_577dup	p.V188_A192dup

Novel mutations are in **bold**; ND, not detectable; NA, not assay.
[a] MCM activity in control lymphocytes = 121 ± 50 pmol/min/mg [19].
[b] MCM activity and genotypes published in Ref. [19].
[c] MCM activity and genotypes published in Ref. [20].
[d] MCM activity and genotypes published in Ref. [18].

Table 3
MUT and *MMAB* polymorphisms.

Exon/ intron	Nucleotide change	Effect on coding region	Frequency in 100 control alleles	Patients that have the polymorphisms
MUT polymorphisms				
Ex 3	c.636A>G	p.K212K	34 (7 homozygous)	2, 4
Ex 8	c.1495G>A	p.A499T	8	1
Ex 9	c.1595G>A	p.R532H	68 (18 homozygous)	1, 2, 4
Ex 12	c.2011A>G	p.I671V	35 (7 homozygous)	2, 4
MMAB polymorphisms				
IVS1	c.135-37_135-38insT	–	18 (2 homozygous)	9
Ex 9	c.716T>A	p.M239K	38 (8 homozygous)	7, 12

Two conclusive pathogenic alleles were identified in each patient, except for patient 9. Patient 9 was heterozygous for c.454G>T (p.E152*) in the *MMAB* gene, which was inherited from the mother [21]. The second pathogenic allele was not detected, although almost 400 nucleotides upstream of the gDNA including the promoter region were sequenced. However, four intron polymorphisms: c.135-37_135-38insT, c.520-96, -128, and -159C>T, inherited from the father were identified in this patient, and not detected in the mother. Analysis of the cDNA showed that the three intron 6 polymorphisms (c.520-96, -128, and -159C>T) were associated with an aberrant transcript, which incorporating 87 nucleotides of intron 6 (c.519 + 91_c.519 + 177) into the sequence (Fig. 1A). This intronic insertion introduces a premature termination codon (PTC) producing a truncated protein losing 77 amino acid residues or the C-terminal 31% of the polypeptide. The analysis of cDNA from patient 9 and her parents yielded two cDNA bands that differed in relative band intensity (Fig. 1B). The smaller fragment was nearly 500 bp, representing the wild-type cDNA confirmed by sequencing. The larger cDNA fragment was nearly 600 bp, containing the extra 87 bp from the exonization of intron 6. The intensity percentage of the larger to smaller fragments in patient 9 was about 70%:30%, whereas that of her father was about 65%:35%, and those of her mother and normal controls (not shown) were approximately 30%:70%.

4. Discussion

In the present study, we report the clinical courses and mutational spectrum of isolated MMA patients from the Thai population. With the categorization into the 3 complementation groups, we can partly predict the phenotypes from the complementation groups and genotypes.

4.1. MUT mutations and phenotypes of the mut patients

The *MUT* mutations contribute to the majority of the mutant alleles in the Thai patients, similar to the data from other populations [5,14]. We could correlate the genotypes with the clinical phenotypes of the *mut* patients. For example, patient 4 had the homozygous missense mutation p.R694W, which has been reported in mut^- patients and known to change a residue located in the cofactor-binding domain [13]. This may explain the relatively mild phenotype and cobalamin responsiveness in this patient. Whereas patient 5 with a severe phenotype including neonatal onset, cobalamin unresponsiveness, recurrent pancreatitis, and RTA, had compound heterozygous mutations: p.G263V and p.D272Gfs*2. Both mutations have not been reported but the first one is located in the substrate-binding N-terminal $(\beta/\alpha)_8$-barrel domain, in which most reported mutations resulted in the mut^0 enzyme subtype [13]. Patients 2 and 6 had homozygous nonsense mutations (p.R31* and p.Q476*, respectively) and severe phenotypes. The p.R31* mutation is located within the mitochondrial targeting sequence, and leads to absence of enzyme [25]. The novel p.Q476* mutation is located in the linker domain, and may produce a truncated polypeptide that contains only the barrel domain and thus could not be active. In addition, the mRNA would be degraded by the nonsense-mediated decay (NMD) system. Patient 3 had the homozygous c.1957-2A>G mutation, which affects splicing of exon 12 [19]. This mutation has been previously reported in the mut^0 patients, but was in compound heterozygous state with different mutations [14]. Our study of the effect of this mutation at the RNA level revealed that it results in 2 aberrant transcripts by activation of cryptic splice sites. The first transcript skips the first 13 nucleotides of exon 12 leading to a reading frame shift and a PTC (p.T653Wfs*15), which triggers mRNA degradation by the NMD system. The second transcript incorporates 76 bp of intron 11 (c.1957-974_c.1957-899) in addition to skipping the first 13 nucleotides of exon 12, which results in the deletion of the first 5 amino acids and insertion of 16 amino acid residues in the exon-12-encoded sequence (p.T653_V657delins16). This will encode a polypeptide with abnormal amino acid sequence in the cofactor-binding domain, but not in degradation of the mRNA by the NMD system. The second transcript may be preferentially transcribed in some tissues, and results in some residual function of MCM. This may explain a relatively mild clinical phenotype in contrast to relatively severe renal complications of this patient.

4.2. MMAA mutations and phenotypes of the cblA patients

The *cblA* subgroup was least common in the Thai patients with isolated MMA. Patient 7 had a known homozygous nonsense mutation (p.R145*), and patient 8 had a novel homozygous missense mutation (p.R98G). The p.R145* mutation is the most common *MMAA* mutation identified in various populations [15,16]. The p.R98G mutation is

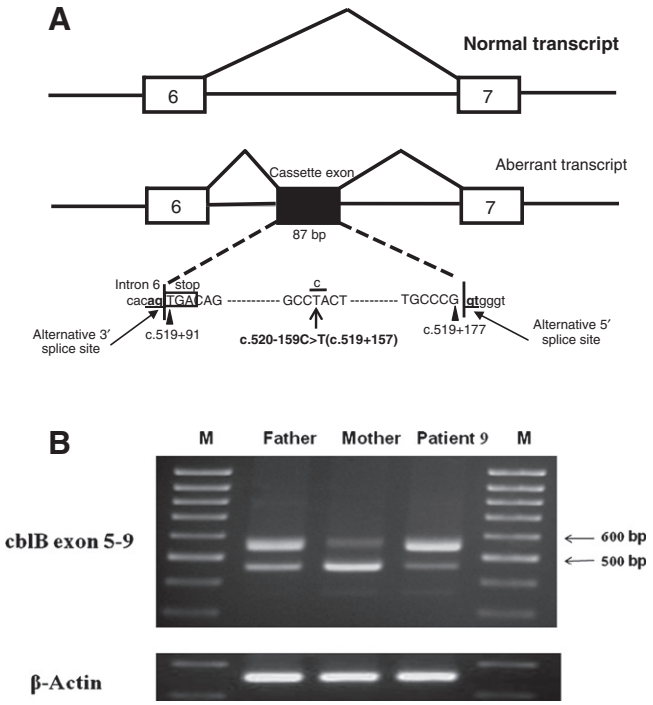

Fig. 1. Alternative splicing of intron 6 of the *MMAB* gene. (A) Schematic presentation of normal and aberrant splicing of intron 6. The genomic locations of exons 6 and 7 and the cryptic exon are indicated by open and black boxes respectively. Uppercase letters indicate sequences of the cryptic cassette exon. Lowercase letters indicate intron 6 sequences. The splice sites used by the cryptic exon are underlined. The vertical lines indicate the cleavage sites. The thick arrow indicates the polymorphism c.520-159 C>T which appears to activate the alternative splice sites (thin arrows). Inclusion of the cryptic exon, starting from c.519 + 91 to c.519 + 177 (arrowheads), introduces a PTC (boxed). (B) RT-PCR products spanning exon 5–9 exhibited larger fragments (nearly 600 base pairs) and smaller fragments (nearly 500 base pairs) with different intensity in patient 9, and her parents. β-Actin was PCR-amplified as a cDNA loading. M, size marker (100-bp ladder).

located in the N-terminal extension which may interact with *MUT* [26]. Overall, they had relatively good outcomes among the isolated MMA patients. This data supports a previous report that found that *cblA* patients had relatively mild phenotypes despite severe genotypes [27]. This could be explained by NMD escape or reinitiation of translation which could maintain potentially functional truncated MMAA protein [27].

4.3. MMAB mutations and phenotypes of the cblB patients

We identified two recurrent *MMAB* mutations in unrelated patients: p.E152* in patients 9 and 10, and p.V188_A192dup in patients 12, 13, and 14. The p.E152* mutation has not been identified in other populations, and might be specific to the Thai population. The p.V188_A192dup mutation has been reported in various populations, and is probably the result of the high mutation rate in a GC-rich region of exon 7 [17]. Three mutations (p.A228Pfs*2, p.F145Lfs*69, and c.585-1G>A) have not been previously reported, and are likely to be pathogenic, because the p.A228Pfs*2 and p.F145Lfs*69 mutations result in frame shifts and truncated proteins, while the c.585-1G>A mutation disrupts the splice acceptor site at the beginning of exon 8, leading to skipping of the entire exon 8, and resulting in the deletion of 20 amino residues (p.V196_R215del). From this study, the phenotypes of the *cblB* patients were relatively more severe than those of the *cblA* patients, but comparable to those of the patients with severe *MUT* mutations. Moreover, patient 12 developed cardiomyopathy, which is a common complication in propionic acidemia, but very rare in isolated MMA. This patient had the most severe phenotype among the *cblB* patients. So far, only 3 patients with isolated MMA, 2 with mut^0 and 1 with *cblB*, have been reported to have cardiomyopathy [28]. This indicates that cardiomyopathy is a complication which could occur in MMA patients, especially those with severe subtypes, and that the cardiac sequelae should be monitored as it is in cases of propionic acidemia [29].

In addition, we identified a novel molecular mechanism in the *cblB* disorder. The three intron 6 polymorphisms (c.520-96C>T, -128C>T, and -159C>T) in patient 9 were associated with an aberrant transcript introducing a PTC, and result in unstable mRNA transcripts that would be degraded by the NMD system. Thus, these intron polymorphisms could account for the abnormal phenotype in this patient. However, these intron polymorphisms were found at high frequency in the normal control alleles (24% in control alleles with 5 homozygous individuals) [21]. It is clarified by the finding of the SNP: rs2287180 (http://www.ncbi.nlm.nih.gov/SNP), the same variant as the c.520–159C>T, which may create exon splicing enhancer (ESE) sequences [30]. Analysis by the ESE finder 3.0 (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home) [31] revealed that this polymorphism increases the number of serine arginine (SR) protein binding sites overlapping the variant nucleotide from 2 in the standard sequence to 4. The polymorphism could activate alternative cryptic splice sites, and a cryptic cassette exon (Fig. 1A). The study of the cDNA revealed that this alternative transcript is present in a small amount in normal controls, but carriers of this polymorphic allele, such as patient 9 and her father, produce more of this alternative transcript than the wild-type one. A similar finding has been observed in another study [32], in which the heterozygote of this polymorphism had a 1.5-fold decrease in total *MMAB* expression compared to the wild-type homozygote. Thus, it is plausible that the intron 6 polymorphism alone may not be deleterious enough to cause a pathogenic effect, but may become a hypomorphic mutant allele if it is in a compound heterozygous state with a null mutant allele. This explains why patient 9 has a relatively mild phenotype compared with patient 10 who has the same null mutation but in a homozygous state. A similar genetic mechanism is found in hemoglobin E (HbE), which results from a G → A substitution in codon 26 of the β-globin gene [33]. This change activates a cryptic splice site that competes with the normal splice donor site, and normally processed RNA is decreased. Although HbE homozygotes are clinically asymptomatic, individuals who are genetic compounds of the HbE mutation and various β-thalassemia alleles (HbE/β-thalassemia) have abnormal phenotypes that are largely determined by the severity of the other allele.

In summary, the clinical and molecular studies of the Thai patients provide additional knowledge of isolated MMA. From our study, the complementation groups and genotypes could predict the phenotypes and clinical outcomes of the patients. In addition, we propose that the common intronic polymorphism in association with the null allele in the *MMAB* gene can lead to an abnormal phenotype.

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