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원 저

한국 메틸말로닌산혈증 환아 10례에서 Somatic Cell 분석과 cobalamin 반응성 연구

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Somatic Cell Analysis and Cobalamin Responsiveness Study in Ten Korean Patients with Methylmalonic Aciduria

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Purpose: Isolated methylmalonic acidemia (MMA) is an autosomal recessive inherited disorder of propionate metabolism. There are two subtypes of *MMUT* gene defects. *Mut*^{0} represents complete loss of methylmalonyl-CoA mutase (MCM) activity while *mut*- is associated with residual MCM activity, which can be stimulated by hydroxocobalamin (OHCbl) supplementation. The objective of this study is to investigate cobalamin responsiveness and mutations present in Korean MMA population.

Methods: We evaluated 10 MMA patients using somatic cell complementation analysis on their fibroblasts to measure MCM activity and vitamin B_{12} responsiveness for the optimal treatment. *MMUT* gene was sequenced to identify the MMA mutations.

Results: For all patients, the incorporation of [^{14}C]-propionate was low, and there was no response to OHCbl. The incorporation of [^{14}C]-methyltetrahydrofolate and [^{57}Co]-CNCbl fell within the normal range. There was adequate synthesis of methylcobalamin while the synthesis of adenosylcobalamin was low. The complementation analysis showed all patients were mut^0. The sequence analysis identified 12 different *MMUT* mutations, including 2 novel mutations, p.Gln267Ter and p.Ile697Phe, were identified. All the patients in this study had neonatal onset of symptoms, belonged to mut^0 complementation class, and as a result, showed no cobalamin responsiveness.

Conclusion: No Korean MMA patient showed cobalamin responsiveness.

Key words: Methylmalonic acidemia, Methylmalonyl-CoA mutase, Mmut, Vitamin B12, Enzyme deficiency

Introduction

Isolated methylmonic acidemia (MMA) is an autosomal recessive inherited disorder of propionate metabolism that is caused by mutations in

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MMUT, MMAA, MMAB, MCEE, or *MMADHC* genes [1,2]. Pathogenic *MMUT* mutations lead to deficient activity of methylmalonyl-CoA mutase (MCM), mitochondrial apoenzyme responsible for the isomerization of L-methylmalonyl-CoA to succinyl-CoA. In this type (mut^0) of MMA, patients have virtually undetectable levels of MCM activity and do not show cobalamin responsiveness. *MMAA* (4q31.1-2), *MMAB* (12q24.1), *MCEE* (2p13.3), and *MMADHC* (2q23.2) genes are respectively associated with *cblA*, *cblB*, epimerase, and *cblD* variant, 2 of the mut^- subtype where are defected in synthesis of adenosylcobalamin (AdoCbl), results in MMA. Unlike patients with mut^0, those with mut^- have residual MCM activity, which can be stimulated by hydroxocobalamin supplementation.

Patients with isolated MMA present with vomiting, hypotonia, respiratory distress, neutropenia, acute ketoacidosis, and hyperammonemia. Mut^0 patients have earlier onset of symptoms, more severe clinical presentations and higher mortality than mut^- patients[2].

Cobalamin incorporation study is a reliable method to diagnose and differentiate the *mmut* disorders[3]. In this study, we evaluated 10 clinically and biochemically diagnosed Korean MMA patients using somatic cell complementation analysis to investigate *mmut* enzyme activity and vitamin B_{12} responsiveness for the optimal treatment. To better elucidate the spectrum of mutations that cause MMA, the *MMUT* gene was sequenced in 10 patients with mmut MMA.

The objective of this study is to check for cobalamin responsiveness in Korean MMA population and identify genotypes present in the patients diagnosed with MMA.

Materials and Methods

1. Patients

Skin fibroblasts and genomic DNA were obtained from 10 Korean patients with MMA after informed consents from the parents of the patients.

2. Methods

1) Biochemical analyses

Acylcarnitines and amino acids were extracted from dried bloodspots and butylated with stable isotope internal standards, and were introduced into the inlet of a tandem mass spectrometer via a high-performance liquid chromatography. If newborn screening or tandem mass spectrometry results were positive for MMA, the diagnosis was confirmed by urine organic acid analysis (gas chromatography-mass spectrometry) and plasma/urine amino acid analysis (ion exchange chromatography).

2) Enzyme activity and cobalamin incorporation study

Skin fibroblast cell lines of all the patient were referred to the Clinical Research Laboratory, Department of Medical Genetics, McGill University Health Centre.

To measure of MCM enzyme activity, patient and control cell lines were incubated under the condition of [^{14}C]propionate or [^{14}C]methylTHF, in the presence and absence of hydroxycobalamin. At the end of the incubation, the MCM function in intact cells was determined by measuring radioactivity in protein of patient through liquid scintillation counting.

To identify cobalamin incorporation, patient and control cell lines were incubated in medium con-

taining ^{57}Co-labeled cyanocobalamin (vitaimin B_{12}). Cells are harvested and then cobalamin derivatives (hydroxycobalamin (OHCbl), cyanocobalamin (CNCbl), 5'-deoxyadenosylcobalarnin (AdoCbl), and methylcobalamin (MeCbl) were separated by high performance liquid chromatography. Percentage of total recovered cellular cobalamin was calculated.

Complementation test were done followed by Watkins et al[3]. Propionate incorporation (or methylTHF incorporation) was compared in parallel fused and unfused cultures. Incorporation level was increased in mixed fused cultures from different complementation classes, compared to parallel mixed unfused cultures.

3) Molecular study

Sanger and targeted massive pararell sequencing of *MMUT* were used to analyze the genomic DNA of the ten patients who were clinically and biochemically diagnosed.

Genomic DNA was isolated from peripheral leukocytes or skin fibroblasts using QIAmp DNA blood kit (Qiagen, Hilden, Germany) Sanger sequencing were done for twelve exons and their intronic flanking regions of the MMUT gene. Targeted massive parallele sequencing were carried out following by Pupabac et al[4]. All 24 targeted gene were enriched with NimbleGen SeqCap probe hybridization (Roche, Madison WI, USA) and subsequently sequence analyzed with HiSeq2000 (Illumina, Sandiego, CA, USA). Alignment and analyses of the variants during massive parallel sequencing were done by NextGENe software (SoftGenetics, State College, PA, USA).

Results

1. Demographics and Clinical Course

Ten patients (6 males, 4 females) with isolated MMA (10 *mut*^{0}) were clinically evaluated and followed up for 2-21 years. In all patients, cellular enzyme activity and mutation analysis confirmed the biochemical diagnosis (Table 1).

Three patients (Patient 2, 3, and 5) were ascertained by NBS, Patient 8 was diagnosed pre-symptomatically by urine organic acid analysis because his older brother had MMA. Patient 6 was confirmed prenatally by molecular analysis because her siblings had MMA. Five other patients were ascertained by metabolic acidosis and hyperammonemia.

The mean age at diagnosis was 1.4 months (range: 0-6 months). Four patients had gastrostomies because of poor feeding. Only one patient had brain atrophy and basal ganglia injury, and as a result, was wheelchair-bound without ambulation.

All had delayed growth. Four patients (2, 4, 5, and 10) improved growth following the liver transplantation. They also had a decrease of MMA levels (from 1,500-4,000 to 500-1,000 μg/mg of creatinine) even though their total protein intake increased. Patient 2 had complicated by portal vein thrombosis after liver transplantation. Other patient complicated by CMV virus and EB virus infection.

One patient had lost eyesight permanently by optic atrophy. Two patients had recurrent pancreatitis and had hypertension from twenty years of age. All patients showed slowly progressive renal function deterioration and decreased GFR. One patient (case 1) presented was most severe clinical died at 12 years of age.

Table 1. Clinical and Biochemical Findings during the Follow-up

Patient #	Age (year)	Sex	Subtype	Age of Dx	Presentation	Clinical course	MMA (μg/mg of creatinine)/ Glycine (μmol/L)	NH3 (μg/dL)/ BUN (mg/dL)/ Cr (mg/dL)	Homocyst(e)ine (μmol/L)	Outcome
1	12	M	*MUT*^{0} early onset	1≥m (NBS)	MA, FTT, SZ, DD, HA, Lethargy, Hypotonia	Tx started late, brain atrophy	3,081/1,004	78–150/ 36–52/ 1.0–1.93/ MCV 91	5.3	Expired
2	2	M	*MUT*^{0} early onset	1≥m (NBS)	VO, FTT	MRI (N), portal vein thrombosis	2,352/1,122	89–162/ 14.3–18.5/ 0.39–0.53	6.7	Liver transplantation
3	7	F	*MUT*^{0}	1≥m (NBS)	AS, VO	MRI (N)	3,056/469	57–103/ 22.5–32.2/ 0.4–0.79	3.18	ND
4	13	M	*MUT*^{0}	2.5≥m	FTT, MA, SZ	MRI (N)	1,710/434	41–108/ 45.4–60.9/ 1.09–1.49	5.5	Liver transplantation
5	3	M	*MUT*^{0} early onset	1≥m (NBS)	Coma, SZ, DD, HA	Recovered from a cardiac arrest Brain MRI (N)	2,425/346	125–1400/ 2.5–15.2/ 0.4–0.45	4.9	Liver transplantation CMV EV infection
6	7	F	*MUT*^{0}	Prenatal (NBS)	VO, HA, MA, Anemia		6,618/605	47–151/ 33–68.4 / 0.77	6.3	ND
7	9	F	*MUT*^{0}	6 m	FTT, HA, MA, Anemia	CRF	2,015/386	73–125/ 37.5–67.1/ 1.74–2.06	7.6	DD
8	20	M	*MUT*^{0} early onset	1≥m	FTT, MA, HA, VO, DD	CRF	2,139/625	64–195/ 39.4–62.3/ 2.00–2.83	8.5	Optic atrophy, recurrent pancreatitis
9	21	M	*MUT*^{0} early onset	1≥m	FTT, MA, HA, VO, DD	CRF, Hypertension	2,934/552	77–166/ 42.7–72.6/ 1.95–2.76	7.4	Recurrent pancreatitis
10	18	F	*MUT*^{0}	1≥m	FTT, MA, HA, VO, DD, Lethargy	CRF, Hypertension	5,816/660	54–185/ 20.3–35.3/ 0.6–1.21/ GOT/GPT 36–69/36–106	3.2	Liver transplantation

Early onset was defined as presentation of first symptoms prior to 30 days of life. Presenting symptoms were obtained through patient record review and/or parental interview.
Abbreviations: BGI, basal ganglia injury; FTT, failure to thrive; HA, hyperammonemia; MA, metabolic acidosis; NBS, newborn screening; Pt, patient; SZ, seizure; DD, developmental delay; OA, optic atrophy; BA, brain atrophy; ND, normal development; AS, asymptomatic; VO, vomiting; Coma; CRF, renal insufficiency.

2. CBC

All patients had anemia (10–12 g/dL, Reference. 11.5–15.0 g/dL). However, MCV values (78.6–91.0 fL, Reference. 80–100 fL) were in the normal range, indicating all patients were normocytic and normochromic.

3. Biochemical test

All patients had elevated C3 carnitine, MMA, and glycine in urine and blood. During metabolic decompensation, metabolic acidosis, increased anion gap, increased blood uric acid, urine ketone, and hyperammonemia were observed in all patients. Patients' biochemical findings are summarized in Table 1.

4. B_{12} Cbl incorprporation

Fibroblasts from all 10 patients had low baseline levels of [^{14}C]-propionate incorporation, which is characteristic of *mut* phenotype. Additionally, all of fibroblasts showed complementation with *cblA* and *cblB* fibroblasts, but not with mut fibroblasts. As a result, all 10 patients were diagnosed with *mut*^{0} MMA.

The incorporation of [^{14}C]-propionate was low, and there was no response or very minimal response to the presence of OHCbl in the culture medium for all the patients. The incorporation of [^{14}C]-methyltetrahydrofolate fell within the reference range. The uptake of [^{57}Co]-cyanocobalamin was slightly high. The synthesis of adenosylcobalamin and of methylcobalamin fell within the reference range. Complementation analysis indicates that these patients fall into the mut complementation class. On the basis of the lack of response of propionate incorporation to the presence of OHCbl in the culture medium, the patients are classified as *mut*^{0}. 257±61 nmol/hour/mg protein, or that of 5, 10 methylene THF reductase 2.3±0.3 nmol/hour/mg protein were comparable to the corresponding values of those activities in control lymphoblasts: 6.7±0.6, 386±36, and 1.8±0.2 nmol/hour/mg protein (Table 2, 3).

Incorporation by patient's cells of radioactivity from [^{14}C]-propionate was normal, but that from [^{14}C]-methylTHF was significantly reduced (Table 3), suggesting a defect of methionine synthesis without an abnormality in the function of methylmalonylCoA mutase.

5. Sequencing

Molecular studies were carried out using targeted massive parallel sequencing for Case 1, 8, 9 and 10 and Sanger sequencing for other pati-

Table 2. Cbl Incorprporation and Cbl Dosage/distribution

Cell line (Patient)#	*Cbl incorprporation	*Cbl dosage/distribution (%)				
	Cobalamin uptake (pg/10^{6} cells)	AqCbl	CNCbl	AdoCbl	MeCbl	Others
1	8.1-8.4	3.7	27.9	5.5	58.3	4.6
Control	22.9-23.5	3.2	10.5	17.7	62.9	5.7
2	15.3-15.4	14.8	6.0	6.6	60.2	12.4
Control	5.2-5.4	13.4	6.3	23.5	42.6	14.2
3	41.5-44.6	5.0	15.5	4.1	61.8	13.6
Control	11.1-11.6	10.0	18.5	17.4	45.5	8.8
4	9.1-9.2	5.6	26.4	4.2	59.3	4.5
Control	22.9-23.5	3.2	10.5	17.7	62.9	5.7
5	6.9-7.3	12.4	12.4	18.3	48.1	8.8
Control	11.5-12.1	10.6	10.3	16.2	53.4	9.5
6	9.8-10.5	9.6	11.6	4.6	55.0	19.2
Control	7.0-7.5	6.7	9.5	17.4	55.4	11.0
7	8.9-10.8	3.1	8.1	8.7	76.0	4.3
Control	11.1-11.6	10.0	18.5	17.4	45.5	8.8
8	9.9	6.4	23.9	4.0	54.9	10.8
Control	14.9	7.2	26.0	12.5	41.1	13.2
9	10.6-11.0	9.2	14.7	3.9	58.5	13.7
Control	23.2-27.1	7.7	16.4	8.8	48.0	19.1
10	6.8-6.8	4.3	14.6	5.2	68.1	7.3
Control	5.9-6.0	3.8	13.7	17.7	57.9	6.9

Table 3. (^{14}C)Propionateand (14C)METHYL-THF

Cell line (Patient)#	(^{14}C)Propionate		(^{14}C)METHYL-THF	
	-OHCbl (nmoles/mg protein/18h)	+OHCbl (nmoles/mg protein/18h)	-OHCbl (pmoles/mg protein/18h)	+OHCbl (pmoles/mg protein/18h)
1	0.7	0.7	606.7	708.1
Control	17.2	17.0	302.4	413.9
2	0.46	0.57	267.3	390.8
Control	15.1	15.1	183.4	231.0
3	1.2	1.4	259.0	299.3
Control	13.0	12.7	211.6	462.9
4	0.5	0.4	500.0	754.0
Control	12.0	12.4	527.3	745.0
5	3.6	3.6	134	183
Control	15.1	15.1	181	231
6	0.4	0.5	214.0	222.6
Control	16.6	19.2	207.4	292.4
7	1.1	1.0	210.6	294.37
Control	13.0	12.7	211.6	462.9
8	0.5	0.6	141.5	153.6
Control	12.0	18.4	165.2	75.0
9	0.4	0.5	141.9	154.0
Control	19.4	17.1	241.4	286.1
0	0.8	0.8	172.0	214.0
Control	7.0	7.0	131.0	316.0

Table 4. Mutation Analyses of MMUT Gene for MMA Patients

Subject	Allele 1	Allele 2
1	c.91C>T (p.R31*)	c.91C>T (p.R31*)
2	c.323G>A (p.R108H)	c.1481T>A (p.L494*)
3	c.799C>T (p.Q267*)	c.1481T>A (p.L494*)
4	c.349G>T (p.E117*)	c.1505_1561del (p.V502fs)
5	c.349G>T (p.E117*)	c.2089A>T (p.I697F)
6	c.322C>T (p.R108C)	c.2179C>T (p.R727*)
7	c.322C>T (p.R108C)	c.2179C>T (p.R727*)
8	c.281G>A (p.G94Q)	c.1105C>T (p.R369C)
9	c.281G>A (p.G94Q)	c.1105C>T (p.R369C)
10	c.682C>T (p.R228*)	c.682C>T (p.R228*)

ents. Genomic DNA was analyzed with the "Cobalamin Metabolism panel and Severe MTHFR Deficiency by Massively parallel Sequencing" test developed at Bayor Miraca Genetics Laboratories. Mutations in *MMUT* were detected in all patients. 8 patients had two heterozygous mutations and 2 had homozygous mutations (Table 4). A total of 12 different mutations in *MUT* were identified in 10 patients; 2 novel mutations, p.Gln267Ter and p.Ile697Phe were identified.

Discussion

1. Clinical

10 Korean patients with isolated MMA were evaluated clinical aspect, biochemical data, B_{12} incorporation study and molecular study. The result of B_{12} incorporation study, including the synthesis of adenosylcobalamin and of methylcobalamin fall into normal range, so MCM activity can be classified *mut*^{0} MMA subgroup in 10 patients.

All ten patients had no enzyme activity and presented very early in life (most within the first month; only two patients presented at around six months) with features including lethargy, which was followed by coma, failure to thrive, recurrent

vomiting, respiratory distress, muscular hypotonia, and hepatomegaly. Most of them had feeding problems, dehydration, seizures and metabolic acidosis as well as hyperammonemia. Their biochemical presentation showed increased glycine, alanine, glutamine in the amino acid analysis. Most also showed anemia (not megaloblastic; normocytic), have potentially life-threatening ketoacidosis, hyperammonemia, mild developmental delay and intellectual deficit. In particular, Patient 1 had frequent seizures, brain atrophy, and severe disability (wheelchair-bound) while Patient 3 had normal growth and development. Those who reached adolescence or adulthood developed renal insufficiency.

Organic acid analysis showed ketone, lactic acid, MMA, methylcitric acid and 3-OH-propionic acid. All the patients treated with glucose and electrolyte IV fluid during metabolic crisis followed by special formula including Propimex or MPA supplemented with low protein food. Essential amino acid (isoleucine and valine) was supplemented. When they grow up, two patients (patient 8 and 9) had recurrent pancreatitis. One patient died at twelve years and never developed motor skills, wheel chair bound, fed through the G-tube.

Most showed some degree of developmental delays, but they were able to attend school and language development was normal. In MMA patients require liver and kidney but all four patients received only liver transplantation. Their protein intake was not restricted, the ammonia and acidosis were maintaining normal range but kidney function were progressively impaired. One patient became blind at ten years of age and two patients had recurring pancreatitis. Two MMA patients were complicated by hypertension after twenty years of age.

The sequence analysis identified 12 different mutations in a number of coding exons, but predominantly in exon 2. 2 novel mutations, c.799C>T (p.Gln267Ter) and c.2089A>T (p.Ile697Phe), were identified in the coding region.

c.799C>T (p.Gln267Ter) and c.2089A>T (p.Ile 697Phe) mutations in this study have not been reported previously in Korean population. The c.322C>T (p.Arg108Cys) and c.323G>A (p.Arg 108His) mutation are relatively common in Korean and Mexican/Hispanic patients[6,10,11]. The c.1105C>T (p.Arg369Cys) mutation identified in patient 8 and 9 has been previously reported in Japanese and American MMA[7,8]. c.1481T>A (p.Leu494Ter) mutation found in patient 2 and 3 was found in compound heterozygous Japanese and Korean patients with MMA[6,9]. The c.349G>T (p.Glu117 Ter) has been found with a high prevalence in Japanese and Korean patients with MMA[6,10,12].

The mutations sepctrum of *MMUT* in the patients with MMA showed no hotspot mutation. All mutations were located almost evenly spanning in all exons. Many *MMUT* mutation in patients with MMA were shared with those of Japanese[6,9,10,12].

In Korea, all the reported MMA patient were *mut*^{0} type of MMA[5,6]. The results of this study showed all 10 patients with MMA were also *mut*^{0} type of MMA. According to these results, there are considered *mut*^{0} type of MMA is most prevalent in Korean population.

요 약

목적: 코발라민(Cobalamin)과 동반되지 않은 독립형 메틸말론산혈증(methylmalonic acidemia)은 프로피오네이트 대사 질환으로 상염색체 열성으로 유전된다. Methylmalonyl-CoA mutase (MCM)효소발현에 관련된 유전자인 *MMUT*에는 유전자 결함에는 두 가지 아형이 있다. Mut^{0}은 효소 활성도가 완전히 없는 것이고 Mut$^-$형은 효소활성도가 저하되어 있지만 hydroxo-

cobalamin (OHCbl) 보충으로 잔여효소의 활성도가 증가될 수 있는 형이다. 본 연구의 목적은 한국인 MMA 환아에서 코발라민의 반응성과 돌연변이를 조사하는 것이다.

방법: 최적의 치료를 위해 MCM 활성도와 비타민 B_{12} 반응성을 측정하기 위하여 섬유 아세포의 체세포 보완 분석을 사용하여 10명의 MMA 환자를 평가했다. MMUT 유전자는 MMA 돌연변이의 염기서열을 확인하였다.

결과: ^{14}C-propionate의 첨가는 OHCbl에 반응이 없는 모든 환자에서 낮게 나타났다. ^{14}C-methyltetrahydrofolate와 ^{57}Co-cyanocobalamin의 투여 후 모두 정상범위 내에 있었다. 아데노 실 코발라민의 합성은 낮지 만 메틸 코발라민의 합성은 적절하였다. 보완 분석 결과 모든 환자들은 *mut*^{0} 유형이었다. DNA 염기서열분석결과에서 2개의 새로운 돌연변이, p.Gln267Ter 및 p.Ile697Phe를 포함하여 12개의 상이한 *MMUT* 돌연변이를 확인하였다. 신생아에서 증상이 나타나며 mut^{0} 형인 MMA 환자 10례 모두에서 코발라민 반응을 보이지 않았다.

결론: 본 연구에서는 모든 한국 MMA 환자에서 코발라민 반응을 시험한 결과 음성이었다.

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