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Three Korean Patients with Maple Syrup Urine Disease: Four Novel Mutations in the *BCKDHA* Gene

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Abstract. Maple syrup urine disease (MSUD) is a rare, autosomal recessive disorder of branched-chain amino acid (BCAA) metabolism caused by dysfunction of the multienzyme branched-chain alpha-ketoacid dehydrogenase (BCKDH) complex. Although a few cases of MSUD have been reported in the Korean population, the genetic background of MSUD is not well understood. In this study, we investigated three newborn males who were diagnosed with MSUD using a standard newborn screening test and amino acid analysis. We screened all coding regions of the *BCKDHA*, *BCKDHB* and *DBT* genes for abnormalities using direct sequencing. Changes in these genes are associated with MSUD. For one patient with complex deletion/duplication mutations, we also performed TOPO TA cloning sequencing. Amino acid analysis showed elevated levels of all branched chain amino acids (BCAAs) in all patients. Three patients were either homozygous or compound heterozygous for the *BCKDHA* mutations. Patient 1 was homozygous for c.1036C>T (p.R346C); patient 2 was heterozygous, with c.632C>T (p.T211M) and c.659C>T (p.A220V); and patient 3 had c.1204_1209dupAAACCC (p.L402_P403dup) and c.1280_1282delTGG (p.L427_A428delinsP). Among these mutations, c.1036C>T, c.632C>T, c.1204_1209dup and c.1280_1282del were novel. These patients had no mutations in either the *BCKDHB* or the *DBT* gene. Although this study included only three patients, the five different mutations in these patients may indicate mutational heterogeneity in Korean patients with MSUD. In addition, the *BCHDHA* gene may present a primary target for clinical genetic analysis. To the best of our knowledge, this is the first report of genetically confirmed MSUD in Korea.

Keywords: MSUD, *BCKDHA*, novel mutation, Korean

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Introduction

Maple syrup urine disease (MSUD) (MIM #248600) is a rare (1:185,000), autosomal recessive disorder of branched-chain amino acid (BCAA) metabolism caused by dysfunction of the multi-enzyme branched-chain alpha-ketoacid dehydrogenase (BCKDH) complex [1]. The BCKDH complex is organized into three subunits: dihydrolipoyl transacylase (E2),

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branched-chain alpha-ketoacid decarboxylase (E1) and dihydrolipoamide dehydrogenase (E3). BCKDH catalyzes the oxidative decarboxylation of branched-chain alpha-ketoacids (BCKAs) produced by transamination of the BCAAs leucine, isoleucine and valine [2].

MSUD occurs in five clinical forms that differ in presentation and severity [3]. Classic MSUD is the most common phenotype, affecting about 75% of patients, and typically presents with severe neurological impairment during the neonatal period. Less common phenotypes include the later-onset intermediate type, episodic intermittent type, intermediate-like thiamine-responsive type and E3 deficiency with lactic acidosis [3]. In classic MSUD, the activity of the BCKD complex is less than 2% of normal. Affected infants typically show lethargy, weight loss, metabolic derangements and progressive neurologic signs, which include alternating hypotonia and hypertonia [1].

Genetic abnormalities related to dysfunction of the BCKDH complex are described according to the loci involved: *BCKDHA* (E1-alpha subunit), *BCKDHB* (E1-beta subunit) or *DBT* (E2 subunit) [1-4]. The prevalence of MSUD is about 1/230,000 in Korea [5]. However, previous reports on Korean patients with MSUD did not describe the associated genetic abnormalities [6-9]. In the present study, we examined DNA samples from three Korean newborn males with the classic MSUD phenotype for mutations in the *BCKDHA*, *BCKDHB* and *DBT* genes.

Materials and methods

Patients

The subjects of this study were three Korean neonatal males. The diagnosis of MSUD was based on the standard newborn screening test (NST), which was performed on dried blood spots by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The parents of these patients were not bound by consanguineous marriages. An analysis of three genes associated with MSUD was performed for each subject. We also recorded each patient's age at diagnosis, signs and symptoms associated with MSUD, amino acid profiles in plasma and urine, and radiologic findings. Due to technical limitations, we were unable to measure BCKDH activity directly. Amino acid analysis was performed in plasma and urine using high performance liquid chromatography. Urine organic acids were analyzed using gas chromatography-mass spectrometry.

Genetic analysis

With parental informed consent, peripheral venous blood samples were collected from each of the subjects. Genomic DNA was isolated from peripheral blood leukocytes using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). The exon-coding regions of the *BCKDHA* (9 exons), *BCKDHB* (10 exons) and *DBT* (11 exons) genes, along with their flanking intronic regions, were amplified by polymerase chain reaction (PCR) using primers designed by the authors (sequences available upon request) and a thermal cycler (Model 9700; Applied Biosystems, Foster City, CA, USA). Five-microliter samples of the amplification product were treated with 10 U shrimp alkaline phosphatase and 2 U exonuclease I (USB Corp., Cleveland, OH, USA). Direct sequencing of the DNA was performed using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) with the BigDye Terminator Cycle Sequencing-Ready Reaction Kit (Applied Biosystems). The cDNA nucleotide sequences of the three genes examined were numbered according to their respective GenBank accession numbers: NM_000709.2 for *BCKDHA*, NM_183050.2 for *BCKDHB* and NM_001918.2 for *DBT*.

Our mutation nomenclature follows the recommendations of the Human Genome Variation Society (http://www.hgvs.org/mutnomen/), with nucleotide +1 corresponding to the A of the ATG translation initiation codon. The frequencies of the novel mutations were investigated through the genotyping of 100 control chromosomes. The PCR product of the *BCKDHA* exon 9 in patient 3 was cloned

into a TOPO vector (Cosmo Genetech, Seoul, South Korea) to obtain eight clones, which we attempted to sequence using the BigDye reaction kit and the ABI PRISM 3130 Genetic Analyzer. To avoid misinterpretation of the reading frames, we used both forward and reverse sequencing. We then compared these clones with a control clone of the *BCKDHA* gene.

Results

Clinical findings

Patient 1 was brought to the outpatient clinic on day 12 of life because of abnormal findings on the NST. He was born at 39+5 weeks by vaginal delivery, with a birth weight of 3.02 kg. An LC-MS/MS analysis revealed elevated concentrations of leucine (2179 μmol/L; normal ≤341 μmol/L), valine (584 μmol/L; normal ≤316 μmol/L) and alloisoleucine (87 μmol/L; normal, not detected). The patient had previously been breastfed, but the infant's ability to suckle was poor. His activity decreased and he experienced a seizure-like episode on day 8 of life. When admitted to our hospital, he was feeding poorly, irritable, somnolent, and spastic. A sweet aroma was detected from the patient's urine, similar to that of maple syrup. An amino acid analysis of his serum and urine showed elevated concentrations of the BCAAs isoleucine, leucine and valine. A urine organic acid analysis revealed elevated aromatic amino acid metabolites (4-hydroxyphenyllactate, 4-hydroxy-phenylpyruvate, and N-acetyltyrosine) and azelaic acid, without similar elevations in branched-chain ketoacids and branched-chain 2-hydroxyacids. Plasma ketone body levels were increased to 3043 μmol/L (normal, < 131 μmol/L). The patient had normal levels of aspartate aminotransferase, alanine aminotransferase, glucose, and electrolytes. The ammonia level was elevated to 80.8 μmol/L (normal, 18-46 μmol/L), but there was no hepatosplenomegaly. After switching to BCAA-free formula, the patient's symptoms, including irritability and limb stiffness, improved.

Patient 2 was born at 39+6 weeks by vaginal delivery, with a birth weight of 3.38 kg. He was presented to our hospital on day 18 of life, having had an abnormal NST. An initial screening test for six major inborn errors of

Table 1. Plasma and urine amino acid analyses in patients with MSUD.

Specimen	Metabolites	Reference range	Units	Case 1	Case 2	Case 3
Plasma	Isoleucine	42.5-93.5	μmol/L	310	187	558
	Leucine	90.8-182.6	μmol/L	3664	508	2600
	Valine	169.1-305.7	μmol/L	849	352	934
Urine	Isoleucine	<0.13	mmol/g Cr	0.25	0.1	0.43
	Leucine	0.01-0.19	mmol/g Cr	2.23	0.55	2.21
	Valine	<0.23	mmol/g Cr	0.42	0.28	0.97

Table 2. *BCKDHA* mutations in three Korean patients with MSUD.

Case No.	Gender	Age of presentation (days)	Clinical phenotype	Exon	Mutation analysis of *BCKDHA* gene Nucleotide level	Protein level	
1	M	12	classic	8	c.1036C>T	p.R346C	Novel
					c.1036C>T	p.R346C	
2	M	18	classic	5	c.632C>T	p.T211M	Novel
				6	c.659C>T	p.A220V	Known
3	M	20	classic	9	c.1204_1209dupAAACCC	p.L402_P403dup	Novel
				9	c.1280_1282delTGG	p.L427_A428delinsP	Novel

The nucleotides of the *BCKDHA* cDNA were numbered according to GenBank accession no. NM_000709.2.
cDNA numbering commences from the ATG start codon, where +1 is the A of the ATG translation initiation codon.

metabolism showed slightly elevated leucine (449.80 µmol/L; normal < 381.18 µmol/L). An LC-MS/MS analysis revealed elevated total concentrations of leucine and isoleucine (470 µmol/L; normal <300 µmol/L).

The aspartate aminotransferase, alanine aminotransferase, glucose, and electrolyte levels were normal, although ammonia was elevated to 74.3 µmol/L. Interestingly, his parents reported that their son had the episodic vomiting only during breastfeeding, and a urine organic acid analysis was normal. However, the amino acid analysis confirmed the diagnosis of MSUD, and the patient was started on BCAA-free formula, which resolved the vomiting.

Patient 3 was presented to our hospital with the suspicion of MSUD on day 20 of life. He was born at 39+5 weeks by vaginal delivery, with a birth weight of 3.40 kg. An NST showed elevated leucine (932.1 umol/L; normal < 457.42 µmol/L) and alloisoleucine levels (97.3 µmol/L; normal not detectable). Subsequent urine organic acid analysis demonstrated an elevation in branched-chain 2-ketoacids (BCKAs), such as 2-oxoisovaleric acid, 1-oxo-3-methylvaleric acid and 2-ketoisocaproic acid, and branched-chain 2-hydroxyacids (2-hydroxyisovaleric acid and 2-hydroxy-3-methylvaleric acid). Plasma and urine amino acid analyses are described in Table 1. In patient 3, plasma ketone body levels were increased to 202 µmol/L and ammonia was elevated to 131.3 µmol/L. The aspartate aminotransferase, alanine aminotransferase, glucose, and electrolyte levels were normal. Brain ultrasonography revealed a grade 1 germinal matrix hemorrhage. Given his amino acid analysis, the patient was diagnosed with MSUD and placed on BCAA-free formula, which normalized plasma isoleucine and valine. Plasma leucine levels remained elevated, however, so the patient was given a formula free of leucine as well.

Genetic analysis

We analyzed the *BCKDHA*, *BCKDHB*, and *DBT* genes from the three Korean neonates suffering from MSUD. Among these patients, we identified three mutations and two variations of unknown significance in the *BCKDHA* gene (Table 2). Patient 1 was homozygous for the 1-bp substitution of c.1036C>T in exon 8. This missense mutation caused a change in amino acid sequence from arginine to cysteine at codon 346 (Figure 1). Patient 2 was shown to be a compound heterozygote for the missense mutations of c.632C>T and c.659C>T (Figure 1). The c.632C>T mutation induced an amino acid substitution of methionine for threonine at codon 211 (p.T211M), while the c.659C>T (p.A220V) is a previously described mutation [10]. These two *BCKDHA* mutations occurred at exons 5 and 6, respectively. Patient 3 was a compound heterozygote for the c.1204_1209dupAAACCC and c.1280_1282delTGG mutations, which induced two amino acid duplications (p.Lys402_Pro403dup) and an in-frame deletion (p.L427_A428delinsP), respectively. Except for p.A220V, all variations were novel. We screened 50 healthy normal subjects by direct sequencing, and none of these carried any of the mutations in the *BCKDHA* gene described above. No mutations in either the *BCKDHB* or *DBT* genes were found in the three MSUD patients.

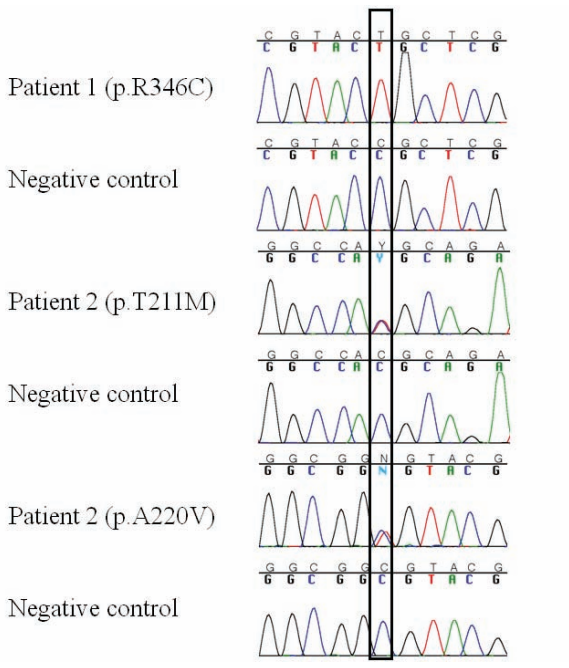

Fig 1. Sequence analysis of the *BCKDHA* gene in Korean patients with MSUD. Patient 1 was homozygous for the c.1036C>T (p.R346C) mutation. Patient 2 showed compound heterozygosity for the mutations c.632C>T (p.T211M) and c.659C>T (p.A220V).

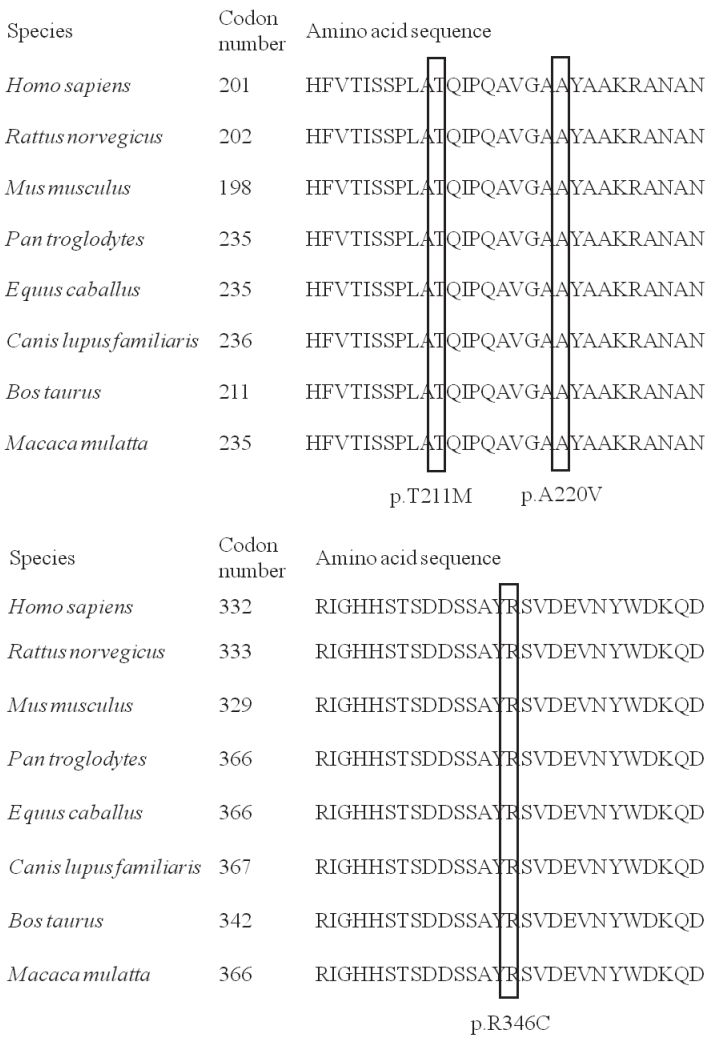

Fig 2. Evolutionary conservation of the amino acid residues for mutant sites. Multiple sequence alignment shows the amino acid sites of three novel mutations (p.T211, p.A220 and p.R346) are highly conserved among different species.

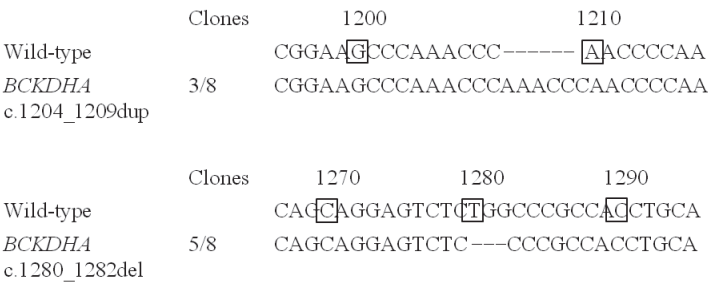

Fig 3. Mutation in the *BCKDHA* exon 9 in MSUD patient 3. DNA sequence traces obtained after TA-cloning. The alignments of the wild-type and mutant exon 9 *BCKDHA* allele sequences are shown. Nucleotides are indicated by capital letters.

The p.T211M, p.A220V and p.R346C mutations of the E1 subunit are most likely responsible for the enzymatic dysfunction of the BCKDH complex because they occur at amino acid residues that are highly conserved across species (including *Rattus norvegicus, Mus musculus, Pan troglodytes, Equus caballus, Canis lupus familiaris, Bos taurus* and *Macaca mulatta*) (Figure 2). We performed a cloning study with patient 3 because he had both a small deletion and a duplication in exon 9 of *BCKDHA*. In patient 3, three of eight clones had the c.1204_1209dupAAACCC, whereas five of eight clones had the c.1280_1282delTGG (Figure 3).

Discussion

Although the measurement of enzyme activity is important for an MSUD diagnosis, our institute lacks the technical capability to assay BCKDH. Instead, we investigated mutational defects in three genes associated with MSUD. Mutational heterogeneity is observed in MSUD [11], and mutations in three specific genes, *BCKDHA*, *BCKDHB* and *DBT*, are associated with approximately 37%, 46% and 13%, respectively, of MSUD cases previously described [12]. In addition, 80% of patients with *BCKDH* mutations are homozygous [12]. Interestingly, only one patient in the current study was homozygous for a mutation, and all three patients had only *BCKDHA* mutations with normal *BCKDHB* and *DBT* genotypes. Within the *BCKDHA* gene, most of the allelic variants (16/20 alleles) that have been detected are clustered within exons 6 and 7 [10]. However, we found only one mutation within these two

exons in our subjects. The rarity of MSUD in the Korean population may be associated with the lack of a founder gene cluster.

Mutations in the human E1b subunit probably induce MSUD through defects in protein structure, because losses of enzyme stability and function correlate well with disease severity [13]. Some *BCKDHA* mutations, such as p.R159W, p.A253T and p.G290R, are associated with a severe form of MSUD. These generally reduce enzyme activity substantially. In a previous study, the mutant *BCKDHA* genes encoding p.N222S or p.G245R produced E1 proteins with residual activity (1.37 and 2.66% of normal activity, respectively), while all other purified mutant E1 proteins tested were inactive [14]. When the significance of a novel sequence variant is unknown, pathogenicity can be supported through family studies, functional analysis, an assessment of its frequency in unaffected controls, conservation across species, and in silico analyses. In this study, we were unable to measure the activity of proteins encoded by genes with the missense mutations; however, we believe that two of the novel missense variations directly cause the loss of BCKDH activity, because the amino acids p.T211 and p.R346 are highly conserved across species. In addition, we did not detect *BCKDHA* mutations at these sites in any of 100 control alleles. The effects of these mutations on protein function await further investigation.

The c.659C>T (p.A220V) mutation, first reported in a Spanish population, is located in the alpha helix region near the EI-beta interface [10]. A p.R346H mutation, induced by the mutation c.1037G>A, has also been reported. This mutation, while close in proximity, is distinct from the c.1036C>T mutation that we report here. There were changes in the hydrophobic compatibility matrix (p.T211M and p.R346C) and the electrical charge compatibility matrix (p.R346C) of the amino acid characteristics [15]. These data indicate that changes in amino acid sequence caused by missense mutations most likely play a causal role in MSUD pathogenesis because the conformational changes they would induce imply changes in BCKDH activity and stability. In-frame deletion and duplication variations such as the c.1280_1282delTGG and c.1204_1209dupAAACCC, do not shift the triplet reading frame, but can lead to synthesis of an abnormal protein. It is unclear that they actually influence protein function, but we think these are mutations rather than polymorphisms, because in-frame deletion mutations in the *BCKDHA* and *BCKDHB* genes are reported [10,16]. In addition, two in-frame variations, c.1280_1282delTGG and c.1204_1209dupAAACCC, are located within the active domain of the *BCKDHA* gene spanning amino acid positions 81 to 444. These findings suggest that in-frame deletions or duplications may alter the structure and function of BCKDH.

Although MSUD is a heterogeneous disorder in the general population, evidence suggests that founder mutations or mutational hotspots may reside in the *BCKDHA* or *BCKDHB* genes [3,17,18]. In Old Order Mennonites from Pennsylvania, MSUD is linked closely to the *BCKDHA* p.Y438N mutation; and some non-Mennonite MSUD patients with this allele are believed to share some degree of Mennonite ancestry [18]. The allelic frequency of the c.117delC mutation in the *BCKDHA* gene among healthy transients from Southern Portugal is estimated at 1.4% and may be attributable to a founder effect [17]. In addition, the p.R183P mutation in the *BCKDHB* gene may represent a founder event within the Ashkenazi Jewish population [3]. From this study of only three patients, we are unable to determine whether a founder mutation exists in the Korean population. However, a founder mutation seems unlikely considering that we found five distinct mutant alleles among three MSUD patients. If it exists, a founder effect or genotype-phenotype correlation with MSUD may be revealed through genetic analysis of a larger sample from the Korean population at large.

Early diagnosis and treatment are essential to reduce mortality of patients with MSUD, and

the value of prenatal testing and/or carrier screening is shown in certain circumstances [19, 20]. In our study, three patients were predicted by NST to have MSUD, which was subsequently confirmed using amino acid analysis and organic acid analysis. These patients responded well to early treatment.

In summary, we identified four novel mutations of the *BCKDHA* gene in three Korean newborns. To the best of our knowledge, this is the first report of MSUD confirmed by genetic analysis in Korea. Though this study included only three patients, our results may reflect the mutational heterogeneity in Korean patients with MSUD. In addition, *BCKDHA* may present a key target for genetic analysis in MSUD.

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