Application of multiplex ligation-dependent probe amplification, and identification of a heterozygous Alu-associated deletion and a uniparental disomy of chromosome 1 in two patients with 3-hydroxy-3-methylglutaryl-CoA lyase deficiency

YUKA AOYAMA1,2, TOSHIYUKI YAMAMOTO3, NAOMI SAKAGUCHI4, MIKA ISHIGE5, TOJI TANAKA6, TOMOKO ICHIHARA7, KATSUAKI OHARA7, HIROKO KOZAN7, YASUTOMI KINOSADA1 and TOSHIYUKI FUKAO4

1Medical Information Sciences Division, United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University, Gifu 501-1194; 2Department of Biomedical Sciences, College of Life and Health Sciences, Chubu University, Kasugai, Aichi 487-8501; 3Tokyo Women’s Medical University Institute for Integrated Medical Sciences (TIIMS), Tokyo 162-8666; 4Department of Pediatrics, Graduate School of Medicine, Gifu University, Gifu 501-1194; 5Department of Pediatrics, Nihon University School of Medicine; 6Department of Pediatrics and Clinical Research, NHO Hokkaido Medical Center, Sapporo, Hokkaido 063-0005; 7Department of Pediatrics, Takamatsu Red Cross Hospital, Takamatsu, Kagawa 760-0017, Japan

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Abstract. Mitochondrial 3-hydroxy-3-methylglutaryl-CoA lyase (HMGCL) deficiency is an autosomal recessive disorder affecting the leucine catabolic pathway and ketone body synthesis, and is clinically characterized by metabolic crises with hypoketotic hypoglycemia, metabolic acidosis and hyperammonemia. In the present study, we initially used PCR with genomic followed by direct sequencing to investigate the molecular genetic basis of HMGCL deficiency in two patients clinically diagnosed with the condition. Although we identified a mutation in each patient, the inheritance patterns of these mutations were not consistent with disease causation. Therefore, we investigated HMGCL using multiplex ligation-dependent probe amplification (MLPA) to determine the copy numbers of all exons. A heterozygous deletion that included exons 2-4 was identified in one of the patients. MLPA revealed that the other patient had two copies for all HMGCL exons. Paternal uniparental isodisomy of chromosome 1 was confirmed in this patient by microarray analysis. These findings indicate that MLPA is useful for the identification of genomic aberrations and mutations other than small-scale nucleotide alterations. To the best of our knowledge, this is the first study describing HMGCL deficiency caused by uniparental disomy.

Introduction

Mitochondrial 3-hydroxy-3-methylglutaryl-CoA lyase (HMGCL; EC 4.1.3.4) deficiency is an autosomal recessive disorder that affects leucine catabolism and ketogenesis. The HMGCL gene, located on chromosome 1p36.1, contains nine exons and spans approximately 25 kb (1). In the majority of HMGCL-deficient patients, the first hypoglycemic crisis occurs before the age of one, while one-third of all cases may have neonatal onset. In acute episodes, laboratory data have shown patients with non- or hypoketotic hypoglycemia with high levels of free fatty acids and severe metabolic acidosis with liver dysfunction and hyperammonemia (2). In Japan, HMGCL deficiency is one of the inborn errors of metabolism screened for in newborns by tandem mass spectrometry. Six Japanese HMGCL-deficient patients, including those previously reported (3) were re-evaluated (2). Among them, three had neonatal onset. Follow-up data showed that two patients experienced hypoglycemic crises even after ten years of age. Developmental delay and epilepsy were noted in two and three patients, respectively (2).

We recently encountered two Japanese HMGCL-deficient patients, whose inheritance patterns of single nucleotide muta-
tions were not consistent with transmission within their families. HMGCL has 23 Alu elements in introns. Recombination between Alu elements results in genomic deletions associated with a number of human genetic disorders (4). Hence, we hypothesized that these patients may have an intragenic deletion by non-equal homologous recombination between Alu elements (5-7). Large homozygous deletions can be suspected by the absence of the deleted exons detected by PCR amplification. However, the detection of heterozygous deletions is difficult using routine PCR amplification of genomic DNA and direct sequencing. Multiplex ligation-dependent probe amplification (MLPA) has been proven to be an efficient and reliable technique for the copy number analysis of each exon in a gene (5,8-10). In the present study, we applied MLPA for the analysis of copy numbers in exons of HMGCL and confirmed mutations in the two patients with HMGCL deficiency.

Patients and methods

Patients. Patient 1 was of the female gender, born to non-consanguineous parents, who presented with hypoglycemia at the age of 2 days. She also experienced hypoketotic hypoglycemic crises at the age of 6, 8 and 13 months. She was diagnosed as having HMGCL deficiency at the age of 13 months by urine organic acid analysis, which detected 3-hydroxymethylglutarate, 3-methylglutaconate and 3-hydroxy-3-methylglutarate. The patient is currently 13 years old. She has epilepsy and developmental delay.

Patient 2 was of the male gender, born to non-consanguineous parents, who presented with vomiting and unconsciousness at the age of 3 months. He was diagnosed as having HMGCL deficiency at the age of 3 months by urine organic acid analysis and blood acylcarnitine analysis. He has experienced ten or more hypoketotic hypoglycemic crises, the last of which was at the age of 4 years. He is currently 8 years old and has achieved normal development. A case report for this patient has been previously published in Japanese (11).

Mutation analysis at the genomic DNA level. The present study was approved by the Ethics Committee of the Graduate School of Medicine, Gifu University, Gifu, Japan. Genomic DNA was purified from peripheral blood samples using Sepa Gene kits (Sanko Junyaku Co., Ltd., Tokyo, Japan). Mutation screening was performed at the genomic level by PCR and direct sequencing, using a set of primer pairs that amplify fragments, including exons and their intron boundaries. The primer sequences are presented in Table Ⅰ.

Establishment of MLPA for the analysis of HMGCL. The MLPA reaction is an efficient and reliable technique for the analysis of exon copy numbers. We designed a pair of MLPA probes for each HMGCL exon, using the human MLPA probe design program (H-MAPD), as previously described (12). The MLPA probe sets for the HMGCL exon are listed in Table II. MLPA reactions were performed according to the manufacturer’s instructions (MRC-Holland BV, Amsterdam, The Netherlands) using 100 ng of genomic DNA, the EK1 MLPA reagent kit and the P200-A1 Human DNA reference kit, which includes reference probes and MLPA control fragments (MRC-Holland BV). The PCR products were separated
Table II. MLPA probes for the HMGCL gene.

| Exon | Product length (base) | Exon length (base) | Primer name | Length | Probe sequence |
|------|-----------------------|-------------------|-------------|--------|----------------|
| 1    | 104                   | 50                | MLPA-HMGCLEX1L | 50     | GGGTTCCCTAAGGGTTGGA<sup>3015</sup> TGGACTGCCCCGGGGGAATTCTGCGCCACAGAT |
|      |                       |                   | MLPA-HMGCLEX1R | 54     | 5087 GGCACGAAAGAGGAAGCGCTCCC CGCCGATTCAGATGGATCTTGCGGCAC |
| 2    | 108                   | 52                | MLPA-HMGCLEX2L | 52     | GGGTTCCCTAAGGGTTGGA<sup>3015</sup> CACCTCTCTCTAATGGGCAATCAGGAGATCAGGAGATCTTGCGGCAC |
|      |                       |                   | MLPA-HMGCLEX2R | 56     | 9905 GAAAATTTGGAAGTTGATCCCGGAGATGCTCTAGATTTGATCTTGCGGCAC |
| 3    | 112                   | 54                | MLPA-HMGCLEX3L | 54     | GGGTTCCCTAAGGGTTGGA<sup>3015</sup> GAAAGGAGAATCTCCTTTGACAGCAATCAGGAGATCAGGAGATCTTGCGGCAC |
|      |                       |                   | MLPA-HMGCLEX3R | 58     | 12609 TTGTTCTCTCTCTAATGGGCAATCAGGAGATCAGGAGATCTTGCGGCAC |
| 4    | 116                   | 56                | MLPA-HMGCLEX4L | 56     | GGGTTCCCTAAGGGTTGGA<sup>3015</sup> TTAATTGTGGAAGTTGATCTTGCGGCAC |
|      |                       |                   | MLPA-HMGCLEX4R | 60     | 12609 TTGTTCTCTCTCTAATGGGCAATCAGGAGATCAGGAGATCTTGCGGCAC |
| 5    | 120                   | 58                | MLPA-HMGCLEX5L | 58     | GGGTTCCCTAAGGGTTGGA<sup>3015</sup> TTAATTGTGGAAGTTGATCTTGCGGCAC |
|      |                       |                   | MLPA-HMGCLEX5R | 62     | 12609 TTGTTCTCTCTCTAATGGGCAATCAGGAGATCAGGAGATCTTGCGGCAC |
| 6    | 124                   | 60                | MLPA-HMGCLEX6L | 60     | GGGTTCCCTAAGGGTTGGA<sup>3015</sup> TTAATTGTGGAAGTTGATCTTGCGGCAC |
|      |                       |                   | MLPA-HMGCLEX6R | 64     | 12609 TTGTTCTCTCTCTAATGGGCAATCAGGAGATCAGGAGATCTTGCGGCAC |
| 7    | 128                   | 62                | MLPA-HMGCLEX7L | 62     | GGGTTCCCTAAGGGTTGGA<sup>3015</sup> TTAATTGTGGAAGTTGATCTTGCGGCAC |
|      |                       |                   | MLPA-HMGCLEX7R | 66     | 12609 TTGTTCTCTCTCTAATGGGCAATCAGGAGATCAGGAGATCTTGCGGCAC |
| 8    | 132                   | 64                | MLPA-HMGCLEX8L | 64     | GGGTTCCCTAAGGGTTGGA<sup>3015</sup> TTAATTGTGGAAGTTGATCTTGCGGCAC |
|      |                       |                   | MLPA-HMGCLEX8R | 68     | 12609 TTGTTCTCTCTCTAATGGGCAATCAGGAGATCAGGAGATCTTGCGGCAC |
| 9    | 136                   | 66                | MLPA-HMGCLEX9L | 66     | GGGTTCCCTAAGGGTTGGA<sup>3015</sup> TTAATTGTGGAAGTTGATCTTGCGGCAC |
|      |                       |                   | MLPA-HMGCLEX9R | 70     | 12609 TTGTTCTCTCTCTAATGGGCAATCAGGAGATCAGGAGATCTTGCGGCAC |

Left primer sequence (5'→3'), GGGTTCCCTAAGGGTTGGA; right primer sequence (5'→3'), TCTAGATTTGATCTTGCGGCAC. Hybridization sequences are shown in italics. MPLA, multiplex ligation-dependent probe amplification.
by capillary electrophoresis on an ABI 3130xl genetic analyzer (Applied Biosystems, Warrington, UK). GeneMapper v4.0 software (Applied Biosystems) was used to analyze the separated products and to retrieve peak intensities corresponding to each probe in the different samples. Integrated peak areas were exported to an Excel 2003 spreadsheet. Data generated from a combination of the HMGCL synthetic probe mix and the P200-A1 probe mix were intra-normalized by dividing the peak area of the amplification product of each probe by the total area of only the reference probes in P200-A1. Secondly, normalization was achieved by dividing this intra-normalized probe ratio in a sample by the average intra-normalized probe ratio of all reference samples.

Deleteion breakpoint characterization. The region surrounding the deletion from intron 1 to intron 4 in patient 1 was amplified using three primer pairs as follows: fragment A: sense primer (In1s1, 5'-ACGAACGGTGTTAAAGAGGCAACAG-3') located at position g.6421-6445 in intron 1 and antisense primer (Ex5as, 5'-TTGGGTACGCTGCTGCCCTCAAGGA-3') located at position g.16255-16231 in exon 5; fragment B: sense primer (In1s3, 5'-GTGATGATTCCAGGAGGTCAAGGGA-3') located at position g.8701-8725 in intron 1 and antisense primer Ex5as; and fragment C: sense primer In1s3 and antisense primer (In4as1: 5'-GAGAGGCATAGGACAGATTCTCC-3') located at position g.15110-15088 in intron 4 (GenBank accession no. NG_013061).

PCR was carried out for 40 cycles at 94˚C for 1 min, 64˚C for 2 min, 72˚C for 2 min followed by a 5-min extension at 72˚C using Takara r-Taq (Takara Shuzo Co., Ltd., Shiga, Japan) and a Takara PCR thermal cycler. After subcloning into the pGEM-T Easy vector (Promega, Madison, WI, USA), the fragments were sequenced.

Comparative genomic hybridization (CGH) and single nucleotide polymorphism (SNP) microarray analysis. Whole genomic copy number analysis was performed for patient 2 using an Agilent SurePrint G3 Hmn CGH + SNP 180K Microarray kit (Agilent Technologies, Santa Clara, CA, USA), according to the manufacturer's instructions. Genomic DNA extracted from peripheral blood was used as a template. Data were extracted using Feature Extraction version 9 (Agilent Technologies) and the results were visualized using Agilent Genomic Workbench version 6.5 (Agilent Technologies).

Results

Mutation analysis. Mutations were screened at the genomic level using PCR amplification followed by direct sequencing. Patient 1 had a heterozygous c.31C>T (p.R11*) mutation in exon 1, and her mother did not have this mutation (Fig. 1). No mutation in the maternal allele was identified. The DNA of the father was not available. Patient 2 had a homozygous c.242G>A (p.W81*) mutation in exon 3. Genomic analyses of his patients revealed that the father was heterozygous for the p.W81* mutation; however, the mother did not have the p.W81* mutation (Fig. 2). Hence, we hypothesized the following: i) the maternal allele may have a large deletion not including exon 1 in patient 1; and ii) the maternal allele in patient 2 may have a deletion including exon 3.

MLPA analysis of HMGCL. We successfully performed MLPA for HMGCL. Similar patterns of amplification were obtained in three controls, enabling copy number to be evaluated in the patient samples (Fig. 3). In patient 1 and his mother, only one copy of exons 2-4 was present, whereas two copies of all other exons were present, suggesting a large deletion including exons 2-4 in the maternal allele (Fig. 3). Patient 2 and his parents all had two copies of all HMGCL exons (Fig. 3). This means that both copies of exon 3 in patient 2 were from the father. We, therefore, suspected that patient 2 has a paternal uniparental disomy of the HMGCL region.

Determination of breakpoints in patient 1. Long-range PCR amplification using DNA from patient 1 yielded fragments that included an approximate 4-kb deletion (Fig. 4A). A large deletion from g.9326 to g.13806 (4481 bp; NG_013061) was identified. We confirmed that one breakpoint was within an Alu element in intron 1 and the other was in a non-Alu element in intron 4 (Fig. 4B).

CGH and SNP arrays in patient 2. To confirm the copy number and SNP haplotype of the HMGCL region, microarray analysis
using CGH + SNP array was performed for patient 2. In the CGH array, no copy number aberration was detected in the whole of chromosome 1 including the \textit{HMGCL} region, which confirmed the homozygous status of the \textit{HMGCL} mutation (Fig. 5).

Furthermore, a loss of heterozygosity (LOH) for almost all of chromosome 1, where \textit{HMGCL} is located, was revealed. This indicated paternal uniparental disomy of chromosome 1. In conclusion, a homozygous mutation in \textit{HMGCL} was determined to be caused by non-Mendelian inheritance due to paternal uniparental isodisomy of the whole chromosome 1, including the 1p36.1 region.

**Discussion**

In the present study, we investigated the molecular genetic basis of two Japanese HMGCL-deficient patients using standard Sanger sequencing. Although we identified a mutation in each patient, inheritance patterns were not consistent in their families. Human \textit{HMGCL} is an Alu element-rich gene, having 23 Alu elements within 23 kb. We first hypothesized that a heterozygous intragene deletion caused by non-equal homologous recombination between Alu elements was a possible cause of \textit{HMGCL} mutation in these patients. Therefore, we used the MLPA method to detect the copy numbers of each exon in \textit{HMGCL}.

Patient 1 had a heterozygous p.R11* mutation from the father, but a mutation from the mother was not identified by conventional sequence analysis. MLPA revealed that patient 1 and her mother had only one copy of exons 2-4, which was consistent with our hypothesis. However, this large deletion that included exons 2-4 was not caused by non-equal Alu-mediated homologous recombination. One breakpoint was within an Alu-Sx element in intron 1 and in a non-Alu sequence in intron 4, as indicated by the inverted triangle.
There are two types of uniparental disomy, heterodisomy and isodisomy. In heterodisomy, a pair of non-identical chromosomes is inherited from one parent and in isodisomy a single chromosome from one parent is duplicated. Isodisomy is potentially dangerous as it may lead to the duplication of lethal recessive genes, while heterodisomy is essentially benign. Uniparental disomy in humans is mainly caused by meiotic non-disjunction events followed by i) gamete complementation; ii) trisomy rescue; iii) monosomy duplication; and iv) somatic crossing over (13). Our present results from HMGCL gene analysis, MLPA and CGH + SNP arrays indicate that patient 2 had a monosomic duplication of paternal chromosome 1. Partial and whole uniparental disomy of chromosome 1 has been reported in autism, fumarase deficiency, Stargardt disease, Pelizaeus-Merzbacher-like disease, leptin receptor deficiency, rhizomelic chondrodysplasia punctata type 2 and CD45-deficient severe combined immunodeficiency (14-20). This study demonstrates the advantage of using MLPA method for the analysis and identification of such heterozygous gene alterations. The identification of such mutations may facilitate mutation analysis in newly diagnosed patients.

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