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Next generation sequencing of patients with *mut* methylmalonic aciduria: Validation of somatic cell studies and identification of 16 novel mutations

Jordan Chu [a], Mihaela Pupavac [a], David Watkins [a], Xia Tian [b], Yanming Feng [b], Stella Chen [b], Remington Fenter [b], Victor W. Zhang [b], Jing Wang [b], Lee-Jun Wong [b], David S. Rosenblatt [a,*]

[a] Department of Human Genetics, McGill University, Montreal, Quebec, Canada
[b] Department of Molecular Genetics, Baylor College of Medicine, Houston, TX, United States

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ABSTRACT

Mutations in the *MUT* gene, which encodes the mitochondrial enzyme methylmalonyl-CoA mutase, are responsible for the *mut* form of methylmalonic aciduria (MMA). In this study, a next generation sequencing (NGS) based gene panel was used to analyze 53 patients that had been diagnosed with *mut* MMA by somatic cell complementation analysis. A total of 54 different mutations in *MUT* were identified in 48 patients; 16 novel mutations were identified, including 1 initiation site mutation (c.2T>C [p.M1?]), 1 missense mutation (c.566A>T [p.N189I]), 2 nonsense mutations (c.129G>A [p.W43*] and c.1975C>T [p.Q659*]), 2 mutations affecting splice sites (c.753 + 3A>G and c.754-2A>G), 8 small insertions, deletions, and duplications (c.29dupT [p.L10Ffs*39], c.55dupG [p.V19Gfs*30], c.631_633delGAG [p.E211del], c.795_796insT [p.M266Yfs*7], c.1061delCinsGGA [p.S354Wfs*20], c.1065_1068dupATGG [p.S357Mfs*5], c.1181dupT [p.L394Ffs*30], c.1240delG [p.E414Kfs*17]), a large insertion (c.146_147ins279), and a large deletion involving exon 13. Phenotypic rescue and cDNA analysis were used to confirm that the c.146_147ins279 and c.631_633delGAG mutations were associated with the decreased methylmalonyl-CoA mutase function observed in the patient fibroblasts. In five patients, the NGS panel did not confirm the diagnosis made by complementation analysis. One of these patients was found to carry 2 novel mutations (c.433G > A [p.E145K] and c.511A>C [p.N171H]) in the *SUCLG1* gene.

1. Introduction

Mutations in the *MUT* gene (MIM# 609058) resulting in deficient activity of the mitochondrial enzyme methylmalonyl-CoA mutase (MCM; EC 5.4.99.2) are responsible for the *mut* type of methylmalonic aciduria (*mut* MMA; MIM# 251000). MCM is responsible for the isomerization of L-methylmalonyl-CoA to succinyl-CoA, a key reaction in propionyl-CoA metabolism. Propionyl-CoA is generated during catabolism of certain branched chain amino acids (isoleucine, threonine, and valine), odd-chain fatty acids, methionine and cholesterol. In the presence of functioning MCM, it is converted to succinyl-CoA which can enter the TCA cycle. Isolated MMA is caused by a deficiency in either the MCM apoenzyme or the intracellular synthesis of its cofactor, adenosylcobalamin. The latter is secondary to mutations in the *MMAA* (MIM# 607481), *MMAB* (MIM# 607568), and *MMADHC* (MIM# 611935) genes, which are associated with the *cblA* (MIM# 251100), *cblB* (MIM# 251110), and *cblD*-variant 2 (MIM# 277410) disorders, respectively.

The *mut* form of MMA is a rare, autosomal recessive disorder characterized by elevated levels of methylmalonic acid in blood and urine. Patients often present symptoms of metabolic acidosis, failure to thrive, recurrent vomiting, lethargy, hypotonia, and dehydration in the newborn period [1]. Long-term complications include metabolic stroke affecting primarily the globus pallidus [2] and kidney disease, which may progress to end-stage renal failure requiring transplantation [3]. The disorder is divided into two subtypes; fibroblasts from patients with mut^0 have virtually undetectable levels of MCM activity, whereas mut^- cells have residual MCM activity that can be stimulated by hydroxocobalamin supplementation of culture medium. This has been shown to be caused by reduced affinity of the mutant MCM for adenosylcobalamin [4]. In general, mut^0 patients have earlier onset of symptoms, more severe clinical presentations and higher mortality than mut^- patients [1].

The human *MUT* gene is located on chromosome region 6p12.3 and spans 13 exons encompassing 35 kb of genomic DNA [5]. The *MUT* mRNA transcript encodes 750 amino acids which constitute the

* Corresponding author at: Research Institute of the McGill University Health Centre, Glen Site, 1001 Décarie Boulevard, Block E, M0.2220, Montreal, Quebec, H4A 3J1, Canada.
E-mail address: david.rosenblatt@mgcill.ca (D.S. Rosenblatt).

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immature enzyme [6]. Upon entering the mitochondria, the mitochondrial leader sequence (residues 1–32) is cleaved from the immature MCM. MCM structure has been studied in both humans and the bacterium *Propionibacterium freudenreichi ssp shermanii*. The MCM α-subunit of this organism shares 65% of its amino acid sequence with human MCM, and X-ray crystallography has revealed highly conserved domain architecture between the human and bacterial enzymes [7,8]. Human MCM is a homodimer. Each subunit contains an N-terminal extended segment (residues 33–87) which is involved in subunit interaction and precedes the two functional domains of the protein. The N-terminal $(\beta\alpha)_8$ TIM barrel domain (residues 88–422) contains the substrate binding site and the C-terminal $(\beta\alpha)_5$ Rossmann domain (residues 578–750) is the adenosylcobalamin binding domain. The two functional domains are connected by a linker region (residues 423–577). The active MCM holoenzyme contains two adenosylcobalamin molecules, one bound to each subunit.

272 pathogenic mutations in the *MUT* gene have been identified, spanning the entire gene (human gene mutation database HGMD® Professional 2015.4 version as of March 2016). While the majority of the mutations have been reported in only a single family, recurrent mutations exist including c.281G>T (p.G94V), c.1867G>A (p.G623R), and c.2150G>T (p.G717V) in black patients [9], c.349G>T (p.E117*) in Japanese patients [10], c.655A>T (p.N219Y) in Caucasian patients [11], c.671_678dup (p.V227Nfs*2) in Spanish patients [12], c.280G>A (p.G94R), c.322C>T (p.R108C), and c.1022dupA (p.N341Kfs*20) in North American Hispanic patients [9], and c.729_730insT (p.D244Lfs*39), c.1280G>A (p.G427D), and c.1630_1631delGGinsTA (p.G544*) in Asian patients [13].

Somatic cell complementation analysis has been shown to be a reliable method to diagnose *mut* patients and to distinguish the *mut* disorder from the other causes of MMA. In the present study, 53 patients that had been diagnosed as *mut* by somatic cell complementation analysis were studied using a NGS panel targeting *MUT* and 23 other genes associated with cobalamin metabolism.

2. Materials and methods

2.1. Patients

Genomic DNA from 53 patients, diagnosed as *mut* by somatic cell complementation analysis, was extracted using the FlexiGene DNA Kit (Qiagen, Canada). All patient cell lines were referred to the Vitamin B_{12} Clinical Research Laboratory (Department of Medical Genetics, McGill University Health Centre) to rule out an inborn error of cobalamin metabolism. This study was approved by the Research Ethics Boards of the Royal Victoria Hospital, Montreal, Quebec, Canada.

2.2. Biochemical testing and diagnosis

Incorporation of label from [1-^{14}C] propionate into trichloroacetic acid-precipitable macromolecules by cultured patient fibroblasts was used as a measurement of MCM function in intact cells. Fibroblasts from all 53 patients had low baseline levels of [^{14}C] propionate incorporation characteristic of the *mut* phenotype. Additionally, fibroblasts from all 53 patients showed complementation with *cblA* and *cblB* fibroblasts, but not with *mut* fibroblasts. As a result, all 53 patients were diagnosed with *mut* MMA. 44 patients were classified as mut^0 and the remaining 9 as mut^- based upon the responsiveness of cellular MCM activity following the addition of hydroxocobalamin to culture medium. Detailed methods for [^{14}C] propionate incorporation and somatic cell complementation analysis are described in Watkins et al. 2000 [14].

2.3. Gene panel sequencing and data analysis

Genomic DNA from all 53 patients was analyzed with the "Cobalamin Metabolism Panel and Severe MTHFR Deficiency by Massively Parallel Sequencing" test developed at Baylor Miraca Genetics Laboratories. This clinically available NGS-based panel is comprised of *MUT* and 20 other genes associated with cobalamin metabolism or with elevated homocysteine levels. Three additional genes (*AMN*, *CUBN*, and *SLC46A1*) that are not currently included in the clinical test were analyzed in this study. Target sequences of all 24 genes (Table s1) were enriched using custom designed NimbleGen SeqCap probe hybridization (Roche NimbleGen Inc., Madison, WI, USA). The captured sequences include all exons and 20 bp of their flanking intronic regions. DNA template libraries were prepared according to the manufacturer's recommendation. Equal molar ratios of 10 indexed samples were pooled to be loaded onto each lane of the flow cells for sequencing on a HiSeq2000 (Illumina, San Diego, CA, USA) with 100 cycle single-end reads. Raw data in base call files (.bcl format) were converted to qseq files before demultiplexing with CAVAv1.7 software (Illumina Inc., San Diego, CA, USA). Demultiplexed data were processed further by NextGENe software for alignment (SoftGenetics, State College, PA, USA). Average depth of coverage of the NGS analysis was 500–1000×. All exons were covered at sufficient depth. The coverage-based depth analysis using NGS data has been previously reported [15].

2.4. Cellular phenotype rescue studies

Retroviral transduction of the wild-type *MUT* gene into immortalized fibroblasts from patients 7 and 17 was used to confirm that the mutations identified in the *MUT* gene were responsible for the MCM deficiency observed in fibroblasts from these patients. Fibroblasts from a *mut* patient with previously identified mutations, a *cblJ* patient and a healthy individual were used as controls. Wild type *MUT* (pDONR-*MUT* construct from GeneCopoeia, Rockville MD, USA) was cloned into the pBABE retroviral vector using Gateway® Technology (Life Technologies, Burlington, ON). The pBABE-*MUT* retroviral vector was subsequently transiently transfected into a Phoenix amphoteric cell line using the HEPES-buffered saline/$Ca_3(PO_4)_2$ protocol and supernatant was collected (https://web.stanford.edu/group/nolan/_OldWebsite/protocols/pro_helper_dep.html). Patient and control fibroblasts were exposed to the retroviral supernatant in the presence of 4 μg/mL polybrene and incubated in media containing 1 μg/mL puromycin for 4 to 5 days to select for cells that had incorporated the vector. [^{14}C] propionate incorporation was then assessed in the transduced and untransduced cell lines.

2.5. cDNA analysis

Total RNA was extracted from cultured skin fibroblasts from patients 7 and 17 using TRI-Reagent (Sigma-Aldrich, Oakville, ON) according to the manufacturer's protocol. The Superscript III First Strand Synthesis System for RT-PCR (Life Technologies) was used to generate cDNA. PCR amplification of 3 overlapping segments encompassing the entire *MUT* open reading frame was performed using the primers *MUT*-c1F (5′-CGGGGGACGCAGAAGTGCAG-3′), *MUT*-c1R (5′-GGCATCAGCCCTGCTTCCTG-3′), *MUT*-c2F (5′-AACCCTCGACTTCGTGGTGATG-3′), *MUT*-c2R (5′-CAATCTGCCTGTTTCGCACTGAA-3′), *MUT*-c3F (5′-ATGTGCTGCCCGAAGACAAGC-3′), and *MUT*-c3R (5′-CAAGCACCTGAACGGCAGCCT-3′).

3. Results

3.1. Sequencing

Mutations in *MUT* were detected in 48 of 53 (91%) patients. 29 patients had two heterozygous mutations (Table 1), 19 had homozygous mutations (Table 2) and no *MUT* mutations were detected in the remaining 5 (Table 3). A total of 54 different *MUT* gene mutations were identified, including 16 that have not been previously reported in the literature (Fig. 1). The 16 novel mutations include 1 initiation site