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Short Report

Genetic and biochemical approach to early prenatal diagnosis in a family with mut methylmalonic aciduria

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Genetic and biochemical prenatal diagnosis was performed at 11 weeks of gestation in a family with a proband affected by mut methylmalonic aciduria (MMA) and homozygotes for the *MUT* gene c.643G>A (p.Gly215Ser) mutation. Both chorionic villus and amniotic fluid samples were used. The presence of high levels of methylmalonic acid and propionylcarnitine determined by gas chromatography/mass spectrometry and LC/MS/MS analysis, respectively, and the identification of the p.Gly215Ser at a homozygous level in foetal DNA allowed a certain, rapid and early diagnosis. To our knowledge, this is the first mut MMA prenatal diagnosis carried out by genetic and biochemical approach.

C Cavicchi*, MA Donati*, S Funghini, G la Marca, S Malvagia, F Ciani, GM Poggi, E Pasquini, E Zammarchi and A Morrone

Metabolic and Muscular Unit, Department of Paediatrics, University of Florence, Meyer Children's Hospital, Florence, Italy

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Corresponding author:
Prof Enrico Zammarchi, MD, Department of Paediatrics, Meyer Children's Hospital, Via Luca Giordano 13, 50132 Florence, Italy.
Tel.: +39 055 5662482;
fax: +39 055 570380;
e-mail: malmetab@unifi.it

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Mut methylmalonic aciduria (mut MMA) (MIM 251000), caused by deficiency of the apoenzyme methylmalonyl-CoA mutase (MCM) (E.C. 5.4.99.2), is an autosomal recessive inborn error of metabolism. MCM is a mitochondrial enzyme that requires adenosylcobalamin as a cofactor and catalyses the isomerization of L-methylmalonyl-CoA to succinyl-CoA.

Mut MMA shows a wide clinical spectrum, ranging from severe acute neonatal onset with early death to late onset mild forms. Biochemical cell studies delineate two phenotypic variants of MCM deficiency: mut^0 with no detectable enzymatic activity and mut^- with residual mutase activity (1).

To date, about 100 mutations have been reported in the human *MUT* gene-encoding MCM (2–4).

The mature enzyme, a homodimer, has three functional domains: N-terminal domain involved in the dimerization of two monomers, $(\alpha/\beta)_8$ barrel domain with the CoA-binding site and $(\alpha/\beta)_5$ C-terminal domain with the cobalamin-binding site (5).

Prenatal diagnosis in MMA has been performed by determination of methylmalonate in amniotic fluid (AF) and maternal urine, by measuring the mutase reaction and cobalamin metabolism in cultured amniocytes, by studies of [^{14}C]-propionate incorporation and by the assay of MCM activity in chorionic villi (CV) (6). Acylcarnitine analysis for MMA prenatal diagnoses has also been reported (7–9).

Here, we report the first concurrent molecular and biochemical approach to prenatal diagnosis at 11 weeks of gestation in a family with a proband affected by neonatal onset mut MMA.

*The authors contributed to this work equally.

Materials and methods

Case report

The male proband was born at term after an uneventful pregnancy and delivery, from healthy non-consanguineous Italian parents, although both originate from a small village in Central Italy. After 3 days, he showed hypotonia, lethargy, poor feeding, metabolic acidosis (pH 7.07, $PaCO_2$ 15 mmHg, PaO_2 136 mmHg, HCO_3^- 4.2 mmol/l, base excess (BE) −26.3 mmol/l) with increased anionic gap (32 mmol/l), hyperammonemia (1232 μg/dl), hyperlactacidemia, hyperglycemia, pancytopenia and ketonuria. To reduce the ammonia level, we started the emergency treatment with arginine hydrochloride and peritoneal dialysis. Increased levels of glycine were detected by plasma amino acids analysis. Urinary organic acids, analysed by gas chromatography/mass spectrometry (GC/MS), showed high levels of methylmalonic acid (1500 mmol/mol creat, n.v. < 2 mmol/mol creat). These data led to a diagnosis of MMA. Clinical picture and biochemical findings rapidly improved with high caloric intake, protein-restricted diet and the administration of hydroxocobalamin (OHCbl), L-carnitine and metronidazole.

A very low uptake of [^{14}C]-propionate and no response to OHCbl in the culture medium were observed on the proband's cultured fibroblasts. Complementation analysis and the lack of response to OHCbl classified the patient as mut^0.

Mutation analysis

Mutation analysis was performed after the parents' informed consent had been obtained. The proband's total RNA was isolated from peripheral blood with the TRIzol reagent (Life Technologies, Rockville, MD), and cDNA was generated using Display THERMO-RT (Eppendorf, Hamburg, Germany). The proband's and his parents' genomic DNA was isolated from whole blood, and foetal genomic DNA from CV, amniocytes and foetal skin fibroblasts using QIAamp DNA kit (Quiagen, Hilden, Germany). Specific primer sets were designed to amplify MUT cDNA and genomic DNA. The exon 3 polymerase chain reactions (PCR) product from genomic DNA was obtained using the primers 5′-AGTAAATCATTTTACCTTGATTC-3′ and 5′-CCTACATTCAAGGAACTATAG-3′. The amplification conditions were denaturation at 94 °C for 3 min, followed by 28 cycles (94 °C for 30 s, annealing at 53 °C for 30 s, extension at 65 °C for 4 min) with a final extension step at 65 °C for 7 min. PCR products were checked on a 1.5% agarose gel, excised and purified using the QIAquick gel extraction kit (Quiagen). Mutation detection was performed by nucleotide sequencing on an ABI PRISM 310 genetic analyzer using BigDye terminator chemicals (Applied Biosystems, Foster City, CA). Digestion of amplified PCR products by KpnI restriction enzyme (Roche Diagnostics, Basel, Switzerland) was performed according to manufacturer's instructions.

Metabolite analysis

Quantification of methylmalonic acid, as trimethyl-silyl derivative after solvent extraction (10), was performed on AF in GC/MS (6890/5973, Hewlett-Packard, Palo Alto, CA) by stable isotope dilution with selected ion monitoring (SIM) (11).

Quantitative assay of acylcarnitine was performed by tandem mass spectrometry (LC/MS/MS) using an Applied Biosystems-Sciex API 4000 triple-quadrupole mass spectrometer equipped with a TurboIonSpray source (Applied Biosystems-Sciex, Toronto, Canada). After methanol extraction, acylcarnitines were analysed as butyl-esters derivatives as previously reported (12).

Results and Discussion

Sequencing analysis, performed on the proband's full-length MUT cDNA, identified a single suspected genetic lesion, c.643G>A. This nucleotide change will cause a p.Gly215Ser amino acid substitution. Moreover, the known polymorphisms c.636A>G (p.Lys212Lys) (13), c.1595A>G (p.His532Arg) and c.2011G>A (p.Val671Ile) (14) were detected at cDNA level. The proband's genomic DNA sequence analysis of all MUT exons and intron-exon junctions confirmed the presence of the c.643G>A (p.Gly215Ser) mutation at a homozygous level in exon 3. This mutation was detected at a heterozygous level in both parents. The presence of the polymorphisms c.636A>G (p.Lys212Lys) in exon 3, c.1595A>G (p.His532Arg) in exon 9 and c.2011G>A (p.Val671Ile) in exon 12 was also confirmed in the proband's genomic DNA. These polymorphisms were present in parents' *MUT* gene and were inherited at a homozygous level by the proband. Based on these data, the existence of a common ancestor can be hypothesized.

The c.643G>A transition removes a natural KpnI restriction site in the corresponding amplified PCR product. This was used to further confirm the c.643G>A substitution in the proband

and in both his parents and to screen 100 control DNAs to exclude the possibility of a benign polymorphism. The c.643G>A (p.Gly215Ser) substitution was not detected in any control, suggesting it is a disease-causing mutation (15).

The p.Gly215Ser mutation has recently been reported as a homozygous change in another mut patient. However, clinical data were not described, and fibroblasts were not available to distinguish between mut^0 and mut^- phenotype (2). The identification of c.643G>A (p.Gly215Ser) mutation at a homozygous level in our mut^0 patient correlates this mutation with the mut^0 phenotype.

To date, 57 missense mutations have been identified in the *MUT* gene, and 32 of them occur in the barrel domain. About 47% of the barrel domain missense mutations are found in exon 3, suggesting a basic role of this region in MCM function. From bacteria to man, the MCM glycine 215 is part of a highly conserved amino acid stretch (GTIQNDILKE) (16), and it is also the first residue of the fourth β-strand of the barrel domain (17). Furthermore, due to the main torsion angle (ϕ, ψ), codon 215 can only be occupied by glycine (2). It can be hypothesized that such amino acid change leads to an impaired protein folding. However, the formal proof of causation and mechanism for this mutation will require further biochemical investigations, such as overexpression and kinetic analysis of the mutant enzyme.

As requested by the parents, a prenatal diagnosis was performed in their next pregnancy at 11 weeks of gestation. Both CV and AF samples, collected with family consent, were used to isolate genomic foetal DNA, and an AF aliquot was also used for metabolite determinations. The presence of the p.Gly215Ser amino acid substitution at a homozygous level, detected by sequencing and enzyme restriction analysis, led us to diagnose an affected foetus (Fig. 1).

To compare genetic and biochemical data, specific metabolites were simultaneously determined by GC/MS and LC/MS/MS on AF. Quantification of methylmalonic acid, performed in SIM mode using stable isotope dilution, was 40 μM (n.v. 0–1.9 μM). The level of methylmalonic acid is in agreement with the values reported in affected foetuses [6.8–76 μM (9, 18–20, Dr. Divry P, personal communication)] at 12–20 weeks of gestation. Quantitative assay of acylcarnitines showed an increased level of propionylcarnitine (C3, 9.7 μM, n.v. 0.2–1.6 μM) and an increased ratio of C3/C0 (0.5, n.v. 0–0.1), C3/C4 (10, n.v. 0.9–2), and C3/C16 (71, n.v. 6.1–22.1). The C3 and C3/C4 values detected in the foetal AF sample agree with the values reported for affected foetuses [3.90–11.20 μM and 8.90–15.50 μM, respectively (9)] at 12–17 weeks of gestation. The main data of the case are summarized in Table 1.

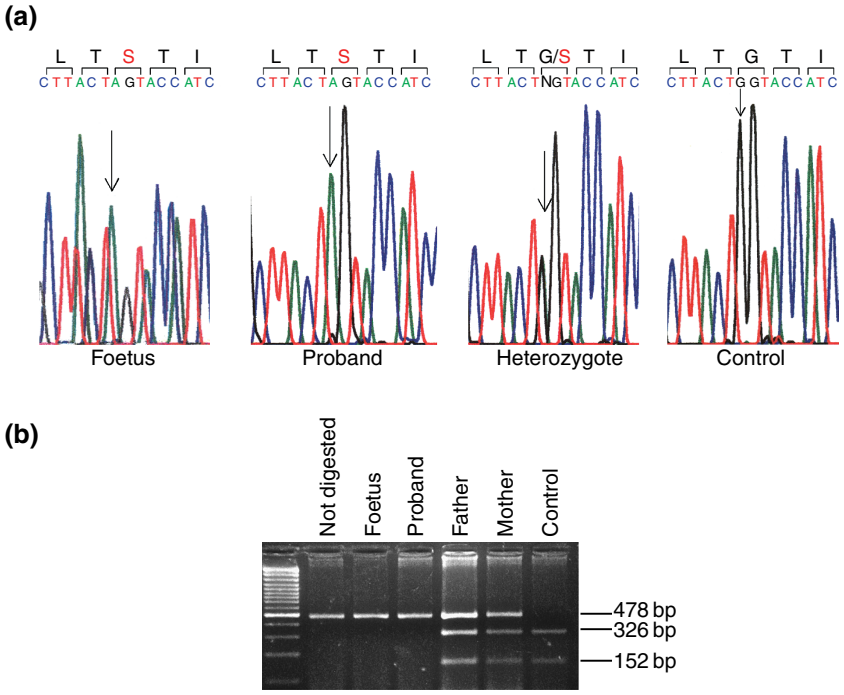

Fig. 1. (a) Partial nucleotide sequence of exon 3 of *MUT* gene. The c.643G>A (p.Gly215Ser) genetic lesion was detected at a homozygous level in the foetus (I) and proband (II) and at an heterozygous status in their parents (III). In panel IV, the sequence of a control is shown. (b) Detection of the p.Gly215Ser mutation by KpnI restriction analysis. The c.643G>A transition eliminates the KpnI site of the 478 bp wild-type polymerase chain reactions product. After KpnI digestion, the wild-type allele gives rise to two fragments of 326 bp and 152 bp. In the presence of the mutation at a heterozygous level, three fragments (478 bp, 326 bp and 152 bp) are obtained. The figure shows the results of the digestion in the foetus, proband, their parents and a normal control.

Table 1a. Salient data of the foetus

	Our foetus	Controls	Affected foetuses
MMA	40	0–1.9	6.8–76
Gestational age	11 weeks	11–12 weeks	12–20 weeks
Reference		Our values ($n = 6$)	9, 18–20, Divry P
C3	9.7	0.2–1.6	3.9–11.20
Gestational age	11 weeks	11–12 weeks	12–17 weeks
Reference		Our values ($n = 8$)	9
C3/C0	0.5	0–0.1	
Gestational age	11 weeks	11–12 weeks	
Reference		Our values ($n = 8$)	
C3/C4	10	0.9–2	8.90–15.50
Gestational age	11 weeks	11–12 weeks	12–17 weeks
Reference		Our values ($n = 8$)	9
C3/C16	71	6.1–22.1	
Gestational age	11 weeks	11–12 weeks	
Reference		Our values ($n = 8$)	
Mutation	p.Gly215Ser (+/+)		

MMA, methylmalonic acid determined in amniotic fluid (μM); C3, propionylcarnitine; C0, free carnitine; C4, butyryl carnitine; C16, palmitoyl carnitine determined in amniotic fluid samples (μM) and their ratios.

Table 1b Salient data of the proband

	Proband	Controls
MMA	1500[a]	<2[a]
Prop − OHCbl	0.55	10.8 ± 3.7
Prop + OHCbl	0.61	10.9 ± 3.5
Mutation	p.Gly215Ser (+/+)	

MMA, methylmalonic acid determined in urine ([a]mmol/mol creat); Prop ± OHCbl, [^{14}C]-propionate uptake (nmoles/mg protein/18 h) with and without hydroxocobalamin (OHCbl) added to the culture medium; (+/+), homozygous state.

Genetic and biochemical data were consistent with the diagnosis of affected foetus, and the pregnancy was terminated at 12 weeks of gestation. After abortion, the p.Gly215Ser mutation was also confirmed on genomic DNA isolated from foetal skin fibroblasts.

Most MMA prenatal diagnostic methods, including methylmalonate and acylcarnitine determinations, MCM activity and [^{14}C]-propionate incorporation assays are performed in the mid-trimester of pregnancy (1, 6). Until now, first-trimester diagnosis of mut MMA had been performed in only a few cases by measuring methylmalonate on AF or by measuring mutase activity and [^{14}C]-propionate incorporation in CV. This latter assay is poorly reliable (21–23), because foetal cell cultures are time-consuming and carries the risk of failure or contamination from maternal cells (24).

The quantitative assay of acylcarnitines and methylmalonate is quick and practical. To our knowledge, at 11 weeks of gestation, no acylcarnitine profiles have been previously described for MMA, while the diagnosis of one affected foetus, by direct quantification of AF methylmalonate (26 μM) (21), has been reported. Our results for methylmalonate and acylcarnitines correlate with the values detected after 12 weeks of gestation for affected foetuses. These data suggest that metabolites concentration is significantly high also at early gestational age as 11 weeks.

The most accurate method for prenatal diagnosis in the first trimester is molecular analysis, but the proband's mutation(s) must be available, and in case of heterozygous foetuses, maternal contamination of the DNA samples should be excluded by marker analysis. To our knowledge, no molecular prenatal diagnoses have yet been reported for mut MMA. Ours is the first to use both biochemical and molecular analysis at 11 weeks of gestation proving to be adequate and reliable.

In conclusion, we would like to stress that mut MMA prenatal diagnosis should be performed using both approaches, together or in a separate manner, with regard to available information and technologies.

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