Mutation Analysis of Methylmalonyl CoA Mutase Gene Exon 2 in Egyptian Families: Identification of 25 Novel Allelic Variants

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Abstract

Methylmalonic aciduria (MMA) is an autosomal recessive disorder of methylmalonate and cobalamin (cbl; vitamin B12) metabolism. It is an inborn error of organic acid metabolism results commonly from a defect in the gene encoding the methylmalonyl-CoA mutase apoenzyme (MCM). Here we report the results of mutation study of Exon 2 of MUT gene (coding MCM residues from 1 to 128) in ten unrelated Egyptian families affected with methylmalonic aciduria. Patients were presented with a wide-anion gap metabolic acidosis. The diagnosis has established by measurement of C3 (propionylcarbamitine) and C3:C2 (propionylcarbamitine/acetocarbamitine) in blood by tandem mass spectrometry, and confirmed by detection of abnormally elevated methylmalonic acid level in urine by gas chromatography-mass spectrometry GC/MS and by isocratic cation exchange "high-performance liquid-chromatography" (HPLC). Direct sequencing of gDNA of the MUT gene exon 2 has revealed a total of 26 allelic variants, ten of which were intronic, four were novel modifications predicted to affect splicing region, eight were located upstream to exon 2 coding region, three were novel mutations within coding region (c.15G>A (p.K55K), c.165C>A (p.N55K) and c.7del (p.R3EfsX14) and the last one was a previously reported mutation c.323G>A.

Keywords: Methylmalonyl CoA mutase; Chromatography; Novel mutation, Egyptian; Single nucleotide polymorphism; Methylmalonic aciduria; Tandem mass spectrometry

Introduction

Methylmalonic aciduria (MMA, MIM# 251000) is an inborn error of organic acid metabolism. It results from a defect in the catabolic pathway of certain branched chain amino acids (valine, isoleucine, threonine and methionine), odd chain fatty acids and cholesterol to TCA cycle passes through propionyl-CoA to methylmalonol-CoA which in turn converted to succinyl-CoA by methylmalonyl-CoA mutase (MCM, EC 5.4.99.2) (Figure 1). MMA is caused by a functional defect in the enzymatic activity of MCM due to defects either in the gene encoding human MCM, causing a serious disorder of propionic acid and methylmalic acid metabolism (termed mut MMA or vitamin B12-unresponsive MMA) [1], or in genes required for the metabolism of its cofactor, 5'-dioxycobalamin (AdoCbl) (called cbl MMA or vitamin B12-responsive MMA) [2]. Recently a few patients have been described with mild MMA associated with mutations of the Methylmalonyl CoA epimerase gene (MCEE) or with neurological symptoms due to (SUCLG1), (SUCLA2) mutations which code for succinate-CoA ligase (SUCL) enzyme complex [3].

The human MUT gene maps to chromosome region 6p12-21.2 (NC_000006.12:49430360-49463328) and has 13 exons spanning over 35 kb of genomic DNA [4,5]. MUT gene is encoded by MCM in the nucleus as a 750 amino acid precursor protein and transported then into the mitochondrial matrix, where its 32 amino acid mitochondrial leader sequence is cleaved [6]. The mature enzyme, 718 amino acids in size, forms a homodimer, each subunit binds 1 molecule of adenosylcobalamin [7]. MCM mitochondrial leader sequence (residues 1–32) is followed by the N-terminal extended segment (residues 33–87), which is involved in subunit interaction. The N-terminal (βα) barrel is the substrate binding domain (residues 88–422) and is attached to the C-terminal (βα) domain (cobalamin binding domain, residues 578–750) by a long linker region (residues 423–577).

Two biochemical phenotypes have been identified in patient fibroblasts with mut MMA; mut+ cells have very low or undetectable levels of MCM activity and mut− cells have residual MCM activity that is increased by the addition of hydroxycobalamin during cell culture, and some of these cells have been shown to have a reduced affinity for adenosylcobalamin [8].

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MMA commonly presents early in life with severe metabolic acidosis, recurrent vomiting, dehydration, hepatomegaly, respiratory distress, muscular hypotonia and progressive alteration of consciousness, probably evolving to overwhelming illness, deep coma and death. Severe combined keto- and lactic acidosis, hypoglycemia, neutropenia, hyperglycinemia and hyperammonemia are the most important laboratory features [9-14]. MMA levels in urine range from 10–20 mmol/mol creatinine in mild disturbances of MMA metabolism to over 20,000 mmol/mol creatinine in severe MCM deficiency [3,15].


Exon 2 is the first coding exons in human MUT gene that codes for MCM amino acids from 1 to 128. It reported among the exon carrying 1048delT and 1706_1707delGGinsTA (p.G544X) in Thai patients [28], c.1595G>A, c.2011A>G in Filipino patients [27], p.E117X in Japanese patients [25], c.655A>C (p.N219T) in Caucasian patients, c.1630-1631delGGinsTA (p.G544X) and c.1280G>A in human MUT of having different disease-causing mutations in MUT gene (exons 2, 3, 6 and 11) [22]. In this study, we reported the results of mutation analysis of exon 2 of MUT gene in eleven Egyptian families who were initially diagnosed by methylmalonic acidemia. We also reported the methods used for diagnosis of MCM, including the biochemical investigations, organic acid analysis by tandem mass spectrometry, gas chromatography-mass spectrometry and isocratic high performance liquid-chromatography.

Patients

About eleven patients (6 males and 5 females) from eleven unrelated Egyptian families, aged from 3 days to 12 years of life, who attended to the Medical Genetics Unit of Ain Shams University Pediatrics Hospital from June 15th 2010 to February 25th 2013 and were suspected Egyptian families, aged from 3 days to 12 years of life, who attended to the Medical Genetics Unit of Ain Shams University Pediatrics Hospital from June 15th 2010 to February 25th 2013 and were suspected of having MMA were included in this study. They were subjected to the screening programs by liquid chromatography-tandem mass spectrometry (LC-MS/MS), gas chromatography-mass spectrometry (GC/MS) and isocratic cation exchange “high-performance liquid-chromatography” (HPLC). All patients were finally diagnosed with MMA except for patient 11 who was initially suspected with MMA for elevated C3 and C3:C2 levels and finally diagnosed with propionic acidemia by GC/MS after the mutation study has been accomplished. However, no enzyme assay was available to confirm the diagnosis. Consanguineous marriages were reported within all families. All reported cases were seen, diagnosed and treated at the Medical Genetics Unit of Ain Shams University Pediatrics Hospital, Cairo, Egypt.

For initial diagnosis, patients’ blood samples were taken by heparin tube, spotted on Whatman filter paper cards (Schleicher and Schuell 903; Dassel, Germany) and left to dry before screening by tandem mass spectrometry. Urine specimens from all studied patients were collected into two plastic laboratory containers and frozen immediately at -20°C until analysis by GC/MS and HPLC. Urine samples from neonates and infants were collected in special sterile plastic bags then transferred into urine containers.

For mutation study, we collected blood specimens from all studied patients in lavender-top tube containing EDTA, immediately centrifuged at 12500 rpm for 10 min, gently rotated for 5 min, then isolated the upper most leukocyte layer, buffy coat, containing DNA with a small portion of plasma and frozen at -20°C for DNA extraction.

The work has been carried out in accordance with the code of ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The work was carried out after the acceptance of parents of the patients and acceptance of the Ethical Committee of the University.

Methods

Metabolite detection

A rapid screening technique of MMA is the analysis of acylcarnitine profiles in dried blood spots by tandem mass spectrometry. Sample preparation and detection procedures were based on methods reported previously [29,30]. Levels of C3 and C3:C2 in dried blood spots were measured by tandem quadrupole mass spectrometry (ACQUITY UPLC® System, Waters associates, northwich, Cheshire, UK) [31] and Acylcarnitines were automatically calculated according to the assigned values of the internal standards using Math Lynx® software. Quality control samples were provided by the Centers for Disease Control and Prevention, Atlanta, GA, USA.

The best way to accomplish the diagnosis is to study urinary nonvolatile organic acid patterns by gas chromatography-mass spectrometry. MMA level in urine was measured by GC-MS (Agilent Technologies Inc., PQ2010). Sample preparation and detection procedures were based on methods reported previously [32].

For initial screening of suspected patients with MMA we used isocratic cation exchange high performance liquid chromatography (HPLC) (supplied by Bio-Rad, Richmond, CA) for determination of organic acids in urine. This technique was previously reported by Bannett et al. [33] and has been used routinely in our department [34].

Mutation detection

DNA was extracted from the patient buffy coats using the G-Spin™ DNA extraction kit (InTRON Biotechnology Inc. Korea). DNA samples of all patients were then amplified and sequenced. PCR primers (Table 1) were used for amplification of a 552 bp genomic region (g.8588-g.9132) of MUT gene (NG_007100.1) exon 2 (g.8635-g.9132) (c.-39-385) and involved: a 385 bp coding region (g.8674-g.9058) for the MCM residues from 1 to 128, a 47 bp upstream open reading frame (ORF) intron (intron ii), as well as an 81 bp downstream ORF intron (intron 2i).

PCR was performed in 25 µl volumes containing 12.5 µl GoTag green master mix (Promega Inc., USA), 1 µl (50 µM) of each primer, 1 µl (25 mM) MgCl2, (Alliance Bio Inc., USA), 1 µl Q-solution (Qiagen Inc., Chatsworth, CA), 5 µl (50 ng) DNA and 4.5 µl nuclease free water (Promega Inc., USA).

The thermocycling program consisted of 5 min denaturation at 95°C, followed by 35 cycles at 95°C for 1 min, 57.7°C for 1 min and 72°C for 1 min and a final extension of 10 min at 72°C in VeritiTM 96-well Thermocycler (Applied Biosystems, Foster City, CA).

PCR products were purified using multiscreen, 96-well PCR clean-up plates (Millipore, Billerica, MA). Sequencing was done in 96-well plates in 10 µl sequencing reactions consisting of 2 µl of PCR product, 0.5 µl of BigDye Terminator Cycle Sequencing Version 3.1 (Applied Biosystems, Foster City, CA), 1.75 µl of 5X sequencing buffer, 5.25 µl of forward primer and 5.4 µl of reverse primer.

<table>
<thead>
<tr>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tbody>
<tr>
<td>5'-TCCCACCCCCCGCTTTCTAAAT-3'</td>
<td>5'-ACAGAGATTAAACCCCCAAGAA-3'</td>
</tr>
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</table>

*Table 1: Exon 2 primers sequences.*
water, and 0.5 μl (50 μM) of sense or anti-sense primer. All amplicons were sequenced in both forward and reverse directions.

For families (2, 3, 5, 6, 7 and 9), products were analyzed on an ABI 3730 DNA Analyzer (Applied Biosystems) and on an ABI 310 automatic sequencer (Perkin Elmer, Foster City, CA, USA) for families (1, 8, 10, and 11).

**Mutation nomenclature and data submission**

The mutation nomenclature is recommended by HGNC (Hugo Gene Nomenclature Committee, http://www.hgvs.org/mutnomen/) and checked by Mutalyzer (https://mutalyzer.nl/check) during submission [35]. The genomic DNA reference sequence from GenBank (NG_007100.1) and the cDNA one (M65131.1) were used in this study, the genomic contig (NT_007592.14) was also used for the genomic DNA sequence. The CDNA numbering commences from the ATG start codon, where +1 is the A of the ATG translation initiation codon. Sequin tool was downloaded from NCBI submission tools http://www.ncbi.nlm.nih.gov/Sequin/gettingstarted.html, and used for submission of all sequencing results to GenBank http://www.ncbi.nlm.nih.gov/ LargeDirSubs/dir_submit.cgi. Genomic, exon 2 and CDS features of MUTE gene were annotated. The accession numbers to the submitted sequences are “KC94079-KC94098” and available at http://www.ncbi.nlm.nih.gov/nuccore/, while the accession numbers to translated proteins are “AGL09917-AGL09935”, and available at protein database http://www.ncbi.nlm.nih.gov/protein/. The detected Single nucleotide polymorphisms (SNPs) and novel allelic variants were then then were then submitted to ClinVar database http://www.ncbi.nlm.nih.gov/clinvar and LOVD [36] https://grenada.lumc.nl/LOVD2/mendelian_genes/home.php.

**MCM structural modelization**

The cloning and sequence analysis of both the human MCM [4,37] and the MCM from Propionibacterium shermanii [38] has revealed the very high amino acid sequence homology (65% identity) between the mature human enzyme and the α-subunit of the P. shermanii enzyme (PDB 1REQ) and human MCM enzyme (PDB: 3BIC). This allowed the construction of a 3D model that satisfies spatial constraints [7]. The human MCM differs in being a homodimer rather than a αβ heterodimer, and it binds 2 adenosylcobalamin molecules per dimer rather than 1. To construct the three-dimensional structure of human MCM, Files were processed using sequence analysis software (PE Applied Biosystems) and were assembled and analyzed using the Phred/Phrap/Consed System [39]. Molecular modeling simulations were performed with the MODELLER 9.11 software [40]. The input to the program is an alignment of the target sequence with the related three dimensional structures (α chain of the Propionibacterium shermanii enzyme (PDB 1REQ) and human MCM enzyme (PDB: 3BIC and 2XIQ)).

**Results**

**Clinical phenotype**

**Patient 1** is the 4th child of consanguineous Egyptian parents which had two dead children from unknown cause. He presented at the 4th month of age with recurrent episodes of vomiting, delayed mental and motor milestones, hyperammonemia, diarrhea, failure to thrive, muscular hypotonia, fever and tachypnea. **Patient 2,** with an older affected sister, was normal till her first year of life when metabolic acidosis, hyperammonemia and cyanosis had developed, accompanied by vomiting and delayed motor and mental milestones. **Patient 3** with family history of dead and affected brothers had presented late at the age of 1 year and 3 months. Laboratory investigations have shown acute metabolic acidosis, hyperammonemia and anemia. **Patient 4** is a 12 months affected child with a family history of two dead members probably with the same condition. He presented with severe hyperammonemia (336.6 μmol/l, reference range <48), disturbed conscious level, tachypnea, loss of sit support and the ability to recognize family members.

**Patient 5** is affected with a neonatal form of the disorder at the first week of age, he presented with delayed mental and physical developments, vomiting, cyanosis, irritability and pyelonephritis. **Patient 6,** with no history of a similar condition, was normal till the age of 1 year and 8 months when he admitted to the PICU with chest infection, bronchial asthma, generalized tonic convolution, acidosis, disturbed conscious level and delayed motor and mental development. **Patients 7 and 8,** also with no history of related conditions, had started their symptoms at the age of 8 months and 10 months respectively, they presented with severe acidosis, lethargy and disturbed conscious level.

**Patient 9** with her older sister, were affected with the neonatal-onset form. They presented with tachypnea, disturbed conscious level, loss of acquired motor and mental development then coma. **Patient 10** with his two older affected brothers, were presented with enlarged liver, otitis media, tonsillitis, fever, persistent vomiting, metabolic acidosis, learning difficulties and delayed developmental milestone manifested by loss of the ability to walk or sit. Coma and PICU admission were reported in the first and the second brothers.

**Patient 11** is affected with a neonatal onset-form of propionic acidemia on the 3rd day after birth. Sequencing analysis was performed accidentally when metabolic screening has detected elevated C3 (35.9 μmol/l) and C3-C2 (0.49) and has suspected with MMA. He presented with jaundice and a severe hyperammonemia reached to 206 μmol/l [reference range<48].

**Biochemical investigations**

Among MMA patients, routine laboratory tests have reported anemia (60%) and severe metabolic acidosis (60%), as well as impaired functions of liver (20%), kidneys (62.5%), and cardiac muscle (10%). Ammonia level was [163.81 ± 101.76 μmol/l] [mean ± standard deviation (SD), reference range <48] indicating that about 54.55% of patients had hyperammonemia prior to treatment. Mean hemoglobin level was [9.43 ± 1.75 g/dl], mean pH was [7.34 ± 0.09], mean anion gap was [15.95 ± 4.05 mmol/l], and the blood urea, estimated on numerous occasions, was varied between 16 mg and 99 mg/dl, mostly over 60 mg/dl, but rose to levels as high as 100 mg/dl.

**Metabolic profiling and HPLC urinary organic acid analysis**

All MMA patients were diagnosed by elevated levels of propionylcarnitine (C3), ratios of C3/acetyl carnitine (C2) in blood, and increased level of methylmalonic acid in urine. Blood levels of C3, C3/C2 were [22.29 μmol/l ± 11.39], [3.16 ± 5.01] (reference range <4, 0.2 consequently). GC/MS Profiling of urine samples of MMA patients has showed high urinary excretion of methylmalonic acid (100%), 3-hydroxypropionic acid (87.5%) and methylcitrate (88.9%) while lactic acid (12.5%), fumaric acid (12.5%), propionic acid (22%), tiglylglycine and propionylglycine were also detected but in a lesser amount.

Figure 2a shows different HPLC profiles of MMA patients and shows an abnormal peak corresponding to MMA, in
addition, smaller peaks of the secondary metabolites of propionate (3-hydroxypropionic and methylcitric acids) were detected. However, methylmalonic acid level decreased consistently after treatment and completely normalized in about eight patients (Figure 2b).

Figure 3 is showing profiles from the propionic acidemia patient before (a) and after (b) management.

**Mutation study**

PCR amplicons of MUT exon 2 for all patients were electrophoresed using 1.5% agarose gel electrophoresis with ethidium bromide staining. All patients had given an amplified exon 2 fragment at 552 bp except in patient 4, where our studies did not record any amplification of exon 2 (Figure 4).

Total mutation study results are represented in genomic level in Figure 5 and in protein level in Figure 6.

Comparison of DNA sequences obtained for the patients with the consensus sequence of the human MCM cDNA (Genebank, accession number M65131.1) has revealed three novel mutations in MUT coding exon 2 (c.15G>A (p.K5K), c.165C>A (p.N55K) and c.7del (p.Arg3GlufsX14)) (Table 2).

Two mutations were identified in more than one patient, a missense mutation consists of C>A transversion at the position 165, c.165C>A.
(p.N55K) and a silent one consists of G>A transition at the position 15, c.15G>A (p.K5K), are likely to be recurrent rather than inherited from a common ancestor and were assessed to be polymorphisms. Families 1 and 11 were compound heterozygous for both mutations c.165C>A and c.15G>A, while patient 5 was heterozygous to c.15G>A polymorphism and patient 9 was heterozygous to the substitution c.165C>A.

The third novel mutation was frame shift c.7del (p.R3EfsX14) in patient 10, which we believed to lead to major amino acid changes and subsequent premature stop codons. Patient 1 was homozygous to a fourth mutation c.323G>A (p.R108H) which previously reported by Acuaviva et al. [26].

Four mutations were predicted to affect the splicing and involved the acceptor/donor consensus splice-site sequences, these mutations are the substitution c.-39-3T>A in family 5, the deletions c.-39-3delT and c.-39-9delT in patient 6 and the insertion c.-39-1-39insA in families 2, 3 and 7 while no significant mutations identified in family 8 (Table 3).

SNPs are dispersed throughout the intronic regions and upstream to exon 2 coding region as well (Figure 5). They are available through the dbSNP of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/clinvar) supplementary table 1. Common polymorphisms were c.-6T>A (in families 1 and 11), c.385+9T>C (in families 1 and 10), c.-37C>A, c.385+29delT and c.385+33A>C (in families 3 and 7) (Figure 5, Table 3).

Phenotype/genotype correlation: Since c.165C>A substitution was heterozygous, it was difficult to correlate the clinical features with the genotype. A common phenotype/genotype correlation of the homozygous mutations p.R108H and p.R3E6X14 in families 1 and 10 respectively, was the clinical severity, but also was variable in both
patients. The hepatic involvement was distinctive clue for the clinical severity of p.R3EfsX14 seen in family 10 as well as the deleted exon 2 in patient 11 from this family. Another clinical feature for p.R108H in family 1 was the neonatal onset.

MCM associated P.N55K modelization study: Partial alignment of MCM amino acid sequence around Asn residue at position 55 in various species (Homo sapiens, P. shermanii, Mus musculus, Escherichia coli, Mycobacterium tuberculosis, and, Caenorhabditis elegans (Figure 7)), indicated that Asn55 is only conserved in man and mouse. Secondary structure motif of MCM molecule (Figure 8) showed that Asn-55 residue lies in the extreme the extreme N-terminus of methylmalonyl-CoA mutase and does not contribute to either the binding of substrate or to the active site but this region is predicted to make extensive contacts with the other subunit that precedes the barrel domain, and a mutant in this region, may prevent the correct assembly of the dimer since homo dimerization is required for MCM activity and that mutation may exert its effect by interfering with homo dimerization and formation of heterodimers. The increased size of the side chain is likely to lead to unfavorable folding. Besides, the introduction of much bulkier hydrophobic Lys residue on the surface of the domain is energetically unfavorable and would disrupt the favorable interactions and lead to unfavorable charge-charge interaction. However, the very low conservative level of the novel missense mutation c.165C>A (p.N55K) within various species, the...

Table 2: Mutations and polymorphisms identified in this study in the coding exon 2 of MUT gene and phenotype/genotype correlation with the homozygous mutations.

<table>
<thead>
<tr>
<th>ID</th>
<th>Gender</th>
<th>Diagnosis</th>
<th>Age of Onset</th>
<th>Presenting Symptoms</th>
<th>C3</th>
<th>C3:C2</th>
<th>Mutation Nucleotide</th>
<th>Amino acid</th>
<th>Variant Remarks</th>
<th>Hom. / Het.</th>
<th>Dom.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>MMA</td>
<td>4 months</td>
<td>Delayed motor and mental development, lethargy, tachypnea, metabolic acidosis, hyperammonemia, vomiting, fever, anemia and diarrhea</td>
<td>30.11</td>
<td>0.47</td>
<td>c.15G&gt;A</td>
<td>p.K5K*</td>
<td>Silent</td>
<td>Hom</td>
<td>ML</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>c.323G&gt;A</td>
<td>p.R108H*</td>
<td>Missense</td>
<td>Hom (B0)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>MMA</td>
<td>3 Days</td>
<td>Delayed motor and mental development, lethargy, tachypnea, metabolic acidosis, hyperammonemia, vomiting, fever, anemia and diarrhea</td>
<td>11.4</td>
<td>0.7</td>
<td>c.15G&gt;A</td>
<td>p.K5K</td>
<td>Silent</td>
<td>Het</td>
<td>ML</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>MMA</td>
<td>6 Days</td>
<td>Tachypnea, disturbed conscious level then coma, loss of acquired motor and mental development, lethargy, hyperammonemia, anemia and admitted into PICU.</td>
<td>26.3</td>
<td>0.67</td>
<td>c.165C&gt;A</td>
<td>p.N55K</td>
<td>Missense</td>
<td>Het</td>
<td>NT</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>MMA</td>
<td>NR</td>
<td>Enlarged liver, otitis media, tonsillitis, fever, developmental regression, loss of motor milestone, vomiting, metabolic acidosis and coma</td>
<td>NR</td>
<td>NR</td>
<td>c.7del</td>
<td>p.R3EfsX14*</td>
<td>Frame Shift</td>
<td>Hom</td>
<td>ML</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>PA</td>
<td>3 Days</td>
<td>Hyperammonemia, jaundice, anemia and NICU admission</td>
<td>35.9</td>
<td>0.49</td>
<td>c.15G&gt;A</td>
<td>p.K5K</td>
<td>Silent</td>
<td>Het</td>
<td>ML</td>
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<td></td>
<td></td>
<td>c.165C&gt;A</td>
<td>p.N55K</td>
<td>Missense</td>
<td>Het</td>
<td>NT</td>
</tr>
</tbody>
</table>

MMA- methylmalonic aciduria, PA- propionic aciduria, PICU- pediatric intensive care unit, NICU- neonatal intensive care unit, NR- not recorded, C3- propionylcarnitine, C3:C2- acetylcarnitine: propionylcarnitine

*aNovel mutations
*bMutation involves CpG dinucleotide

Normal Reference values; C3<4.0 μmol/L, C3:C2<0.30

Table 6: Partial Protein alignment of the amino acid residues (1-128) of MCM for the studied patients , positions of individual mutations are indicated in underlying bold.
heterozygosity, beside its recurrence in non mut MMA patients, (the 11th patient with propionic acidemia), make it very likely pathogenically insignificant and doesn't interfere enzymatic catalysis. Overall, although substitution of Asn55 by a Lys residue involved a change in the size and physical property of the substituted amino acid but it doesn't influence the MCM conformation and activity in our patients. Therefore c.165C>A (p.N55K) is expected to be a frequent heterozygous mutation but parental consanguinity within all studied families, suggests that the previously reported mutation c.323G>A (p.Arg108His). Genetic heterozygosity is high among the identified mutations and the haplotype analysis to study the origin of these mutations has not been performed but parental consanguinity within all studied families, suggests that these mutations were inherited from a common ancestor. Most of the identified mutations were found in family 1, while no significant changes and subsequent premature stop codons, a heterozygous silent mutation c.7del (p.Arg3GlufsX14) was seen in patient 10 which we believed to lead to major amino acid changes and subsequent premature stop codons, a heterozygous silent c.15G>A (p.K55K) mutation was identified in families (1, 5 and 11) and a heterozygous missense one c.165C>A (p.N55K) was reported in three other families.

Discussion

This study highlights some important aspects of methylmalonic aciduria diagnosis in eleven unrelated consanguineous families from Egypt. Diagnostic studies of MMA had established by elevated levels of propionylcarnitine (C3), ratios of C3/acetylcarnitine (C2) in blood by tandem mass spectrometry to all studied patients. GC/MS had confirmed the diagnosis of methylmalonic acidurias to only ten patients (from 1 to 10) by elevated levels of methylmalonic acid in urine, while patient 11 was diagnosed with propionic academia due to elevated propionic acid level in urine.

For initial screening of organic acids in urine we have also used isocratic cation exchange High Performance Liquid Chromatography (HPLC) for qualitative analyses of urine samples from neonates and infants suspected of having organic aciduria. Chromatograms obtained from the studied patients by this method have shown elevated levels of methylmalonic, methylocitrill and 3-hydroxypropionic acids. However, methylmalonic acid in urine was easily detected by this method in the initial attacks of MMA where methylmalonic acid was significantly elevated in urine, but confirmation analysis by GC/MS would still be needed [31].

Among MMA patients, routine laboratory tests have reported hyperammonemia, anemia and severe metabolic acidosis, as well as impaired functions of liver, kidneys, and cardiac muscle.

Initial management involved protein restriction, correction of metabolic acidosis, infection and electrolyte imbalance. MMA or XMTVI milk, carnitine 100 mg/kg/day, depovite injection every day for the first three days then taken every two days, biotin tab 5 mg twice daily and IV fluid according to the patient condition [10,12]. In about eight patients, MMA decreased consistently after treatment; they even returned to normal levels, these approaches match that reported by Hörster et al. [10].

The mutation study involved direct DNA sequencing of the genomic region (g.8588-g9132) of MUT gene exon 2 (g.8588-g9058), as an approach to report common mutations of MUT gene exon 2 in all studied patients including patient 11 who has included before final diagnosis with propionic academia. The sequenced region was a 552 bp and involved exon 2 (g. 8635-g.9058), a 385 bp coding region (g.8674-g.9058) which codes for the MCM residues from 1 to 128, a 47 bp upstream open reading frame (ORF) intron (intron 1i) and an 81 bp downstream ORF intron (intron 2i).

The findings of PCR product were matched with that reported by Worgan et al. [22] since a 552 bp DNA fragment was detected in all patients except in patient 4 who have not shown exon 2 PCR product. This study has revealed a total of 27 variants: eleven of which were intronic, eight were located upstream to exon 2 coding region, three were novel mutations within coding region (located in the mitochondrial leader sequence and in the N-terminal of MCM enzyme), four were novel modifications predicted to affect splicing, and the last one was the previously reported mutation c.323G>A (p.Arg108His). Genetic heterozygosity is high among the identified mutations and the haplotype analysis to study the origin of these mutations has not been performed but parental consanguinity within all studied families, suggests that these mutations were inherited from a common ancestor. Most of the identified mutations were found in family 1, while no significant mutations identified in family 8, and for that, mutation studies to the other mut exons are recommended. The novel mutations identified in the coding region were; a frame shift mutation c.7del (p.Arg3GluX14) was seen in patient 10 which we believed to lead to major amino acid changes and subsequent premature stop codons, a heterozygous silent c.15G>A (p.K55K) mutation was identified in families (1, 5 and 11) and a heterozygous missense one c.165C>A (p.N55K) was reported in three non-related families (1, 9 and 11).

Table 3: Results of mutation study of MUT gene exon 2 in 10 Egyptian Families with MMA and one Egyptian patient with PA.

<table>
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<th>ID</th>
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</table>

Table 3: Results of mutation study of MUT gene exon 2 in 10 Egyptian Families with MMA and one Egyptian patient with PA.

Figure 7: Partial alignment of MCM amino acid sequence around Asn55 is in Homo sapiens, Propionibacterium shermanii, Mus musculus, Escherichia coli, Mycobacterium tuberculosis and Caenorhabditis elegans. (Swiss Prot accession numbers P22033, P11653, P16332, P27253, P71774.1 and Q23381 respectively), open and close boxes represent α helices and 3(10) helices respectively, and the arrow refers to Asn-55 residue which conserved in Human and Mouse. Resource is available at http://www.uniprot.org/align/20130524404TMUYT7Q.

Figure 8: View of the three-dimensional structure of the human methylmalonyl-CoA mutase enzyme models built on the basis of experimental structure of the α chain of the P. shermanii enzyme (PDB 1REQ) and human MCM enzyme (PDB 3BIC and 2XIQ) by Modeller 9.11, showing increased size and steric clash made by hydrophobic positively charged Lys residue located in position 165 in the extreme N-terminal extension.

Authors [16-22,26], the only previously reported mutation in this study was the homozygous mutation c.323G>A (p.R108H) in patient 1 which previously reported white and Korean patients [26]. The highly conserved arginine at position 108 is in the first β-sheet of the N-terminal (βα) barrel and is directly involved in binding the ADP-ribosyl moiety of the CoA ester substrate at the entrance of the substrate channel [7]. Since arginine 108 is important for substrate binding, the p.R108H mutation is likely to be pathogenic.

Previously stated common ethnic mutations in exon 2 were; c.322C>T (p.R108C) in Hispanic patients [23] and p.E117X in Japanese patients [25]. However, the present study has revealed two heterozygous frequent novel mutations c.15G>A (p.K5K) and c.165C>A (p.N55K), possibly common within Egyptian populations.

The c.15G>A (p.K5K), located in the mitochondrial leader sequence, has a silent effect on the transcribed amino acid (Lys residue). It doesn't affect the enzymatic activity or MCM folding therefore c.15G>A is suggested to be a common natural polymorphism.

Homology model of c.165C>A (p.N55K) mutation of human MCM constructed by Modeller 9.11 on the basis of homology with the Propionibacterium shermanii enzyme [7,26] has shown that the N55K mutation is located in the extreme N-terminal and the much bulkier, hydrophobic Lys side chain might hamper the positioning of adjacent helix in the MCM homodimers (due to steric clash), leads to change in N-terminal folding that may interfere with the homo dimerization necessary for MCM activity, but the low conservative level of Asn 55 residue among studied species in the conservation study (H. sapiens, P. shermanii, M. musculus, E. coli, M. tuberculosis, and, C. elegans), the heterozygosity of the mutation and its occurrence in the patient with propionic acidemia, suggested that c.165C>A (PN55K) mutation doesn't interfere the catalytic activity of MCM enzyme in studied patients. However, restriction analysis and mutation studies to the other mut exons would provide a valuable confirmation to the pathogenicity of this mutation and reveal the phenotype-genotype correlations.

Single nucleotide polymorphisms were spread all over the intronic non-coding areas of MUT gene exon 2 and were reported within all families. Mutations that we predicted to affect splicing due to their location in the acceptor/donor consensus splice-site sequences were c.-6T>A (in families 1 and 11), c.385+9T>C (in families 1 and 10), c.-37C>A, c.385+29delT and c.385+33A>C (in families 3 and 7).

Overall, the sequence mutation analysis of the MUT gene exon 2 identified a high proportion of frequent heterozygous mutations within the studied ten Egyptian families. However, the phenotype resulting from compound heterozygosity has not been precisely characterized. However, it would be important to analyze the other MUT exons as well as MMAA, MMAB and MMADHC genes in the patients with only one or no mutations in the MUT gene as it is possible that a mutation in another non-genotyped MUT exons is responsible for the clinical phenotype, or that the MUT deficiency is a part of a general deficiency of mitochondrial enzyme function.
Acknowledgement

We are very grateful to the patients and their families, the laboratory staff and the pediatricians worked in the Medical Genetics Unit of Ain Shams University Pediatrics Hospital who sent DNA samples and provided clinical information and for their cooperation, advice and interpretation of results.

References