



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 which commonly results from a defect in the gene encoding the methylmalonyl-CoA mutase (MCM) apoenzyme. Here we report the results of mutation study of exon 2 of the methylmalonyl CoA mutase (*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 the measurement of C3 (propionylcarnitine) and C3:C2 (propionylcarnitine/acetylcarnitine) in blood by using liquid chromatography–tandem mass spectrometry (LC/MS–MS) and was confirmed by the detection of an abnormally elevated level of methylmalonic acid in urine by using gas chromatography–mass spectrometry (GC/MS) and 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, eight were located upstream to the exon 2 coding region, four were novel modifications predicted to affect the splicing region, three were novel mutations within the coding region: c.15G>A (p.K5K), c.165C>A (p.N55K) and c.7del (p.R3EfsX14), as well as the previously reported mutation c.323G>A (p.R108H).

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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 methylmalonyl CoA which in turn converted to succinyl-CoA by methylmalonyl-CoA mutase (MCM, EC 5.4.99.2) (Fig. 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 methylmalonic acid metabolism (termed *mut* MMA or vitamin B₁₂-unresponsive MMA) (McKusick, 1990), or in the genes required for the metabolism of its cofactor, 5-deoxyadenosylcobalamin (AdoCbl) (called *cbl* MMA or vitamin B₁₂-responsive MMA) (Rosenblatt and Fenton, 2001). 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 (Fowler et al., 2008).

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 (Ledley et al., 1988), (Nham et al., 1990). MCM is encoded by the *MUT* gene 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 (Jansen and Ledley, 1990). The mature enzyme, 718 amino acids in size, forms a homodimer of α and β subunits, each subunit binds 1 molecule of adenosylcobalamin (Thomä and Leadlay, 1996). 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 ($\beta\alpha$)₈ barrel is the substrate binding domain (residues 88–422) and is attached to the C-terminal ($\beta\alpha$)₅ domain (cobalamin binding domain, residues 578–750) by a long linker region (residues 423–577) (Thomä and Leadlay, 1996).

Two biochemical phenotypes have been identified in patient fibroblasts with *mut* MMA: *mut*⁰ 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 (Willard and Rosenberg, 1977).

MMA commonly presents early in life with severe metabolic acidosis, recurrent vomiting, dehydration, hepatomegaly, respiratory distress, muscular hypotonia and progressive alteration of consciousness, probably

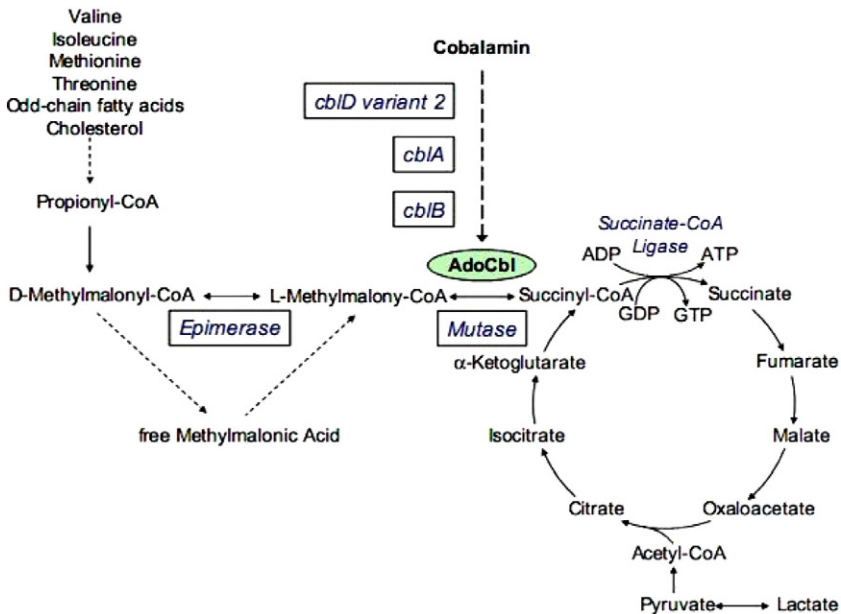


Fig. 1. Metabolic interrelationships of methylmalonic acid, methylmalonyl CoA epimerase, methylmalonyl CoA mutase and other metabolites (Fowler et al., 2008).

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 (Baumgartner and Viardot, 1995; Hörster and Hoffmann, 2004; Hörster et al., 2007; Nicolaides et al., 1998; Ogier de Baulny et al., 2005; van der Meer et al., 1996). MMA level in urine ranges from 10 to 20 mmol/mol creatinine in mild disturbances of MMA metabolism to over 20,000 mmol/mol creatinine in severe MCM deficiency (Fowler et al., 2008), (Boulat et al., 2003).

Various studies have identified different disease-causing mutations in the human *MUT* gene (Acquaviva et al., 2005; Cavicchi et al., 2001; Heptinstall et al., 1999; Jung et al., 2005; Ledley and Rosenblatt, 1997; Martínez et al., 2005; Mikami et al., 1999). Some of these mutations have been reported as mutation-specific populations, including the following: c.322C>T (p.R108C) in Hispanic patients, c.1630–1631delGGinsTA (p.G544X) and c.1280G>A (p.G427D) in Asian patients (Worgan et al., 2006), p.G717V in black patients (Adjalla et al., 1998a), p.E117X in Japanese patients (Ogasawara et al., 1994), c.655A4T (p.N219Y) in Caucasian patients (Acquaviva et al., 2001), c.1595G>A and c.2011A>G in Filipino patients (Silao et al., 2009), 1048delT and 1706_1707delGGinsTA (p.G544X) in Thai patients (Champattanachai et al., 2003), and c.671–678dup in Spanish patients (Martínez et al., 2005).

Exon 2 is the first coding exon in the human *MUT* gene that codes for MCM amino acids from 1 to 128. It has been reported to be one of the *MUT*–exons that carry the majority of disease-causing mutations (exons 2, 3, 6 and 11) (Jung et al., 2005). In this study, we reported the results of mutation analysis of exon 2 of the *MUT* gene in eleven Egyptian families who were initially diagnosed with methylmalonic acidemia. We also reported the methods used for the diagnosis of MMA, including the biochemical investigations, organic acid analysis by tandem mass spectrometry, gas chromatography–mass spectrometry and isocratic high performance liquid-chromatography.

Patients

Patients included in this study were eleven members of eleven unrelated Egyptian families, who were suspected of having *mut* MMA: 6 males and 5 females, 3 days to 12 years of age. All reported cases were seen, diagnosed and treated in the Medical Genetics Unit of Ain Shams University Pediatrics Hospital, Cairo, Egypt, from June 15, 2010 to February 25, 2013. Consanguineous marriages were reported within all families.

For initial diagnosis, patients 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). Patients' blood samples were taken by heel stick, 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 and then transferred into urine containers.

For mutation study, we collected blood specimens from all studied patients in a lavender-top tube containing EDTA, immediately centrifuged at 12,500 rpm for 10 min and gently rotated for >5 min, and then we isolated the uppermost leukocyte layer, buffy coat, containing DNA with a small portion of plasma and frozen at -20°C for DNA extraction.

All patients were finally diagnosed with MMA except patient 11 who was initially suspected with MMA for elevated C3 and C3:C2 levels and finally diagnosed with propionic acidemia by using GC/MS after the mutation study has been accomplished. However, no enzyme assay was available to confirm the diagnosis.

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 the 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 using tandem mass spectrometry. Sample preparation and detection procedures were based on methods reported

previously (Han et al., 2005); (Zytovicz et al., 2001). 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) (Han et al., 2007). 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 using gas chromatography–mass spectrometry. MMA level in urine was measured by GC–MS (Agilent Technologies Inc., QP2010). Sample preparation and detection procedures were based on methods reported previously (Kuhara, 2002). We also used isocratic cation exchange high performance liquid chromatography (HPLC) (supplied by Bio-Rad, Richmond, CA) for screening of methylmalonic acid in urine. This technique was previously reported by Bannett and Bradey (1984) and has been used routinely in our department for initial screening of suspected patients with organic aciduria (Ghoraba et al., 2014).

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 using the following primers: forward primer: 5′-TCCCACCCCTCTTCTAAAT-3′, reverse primer: 5′-ACAGAGATTAACCCCCAAAAA-3′ (Worgan et al., 2006). 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 4.50 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 a Veriti 96-well thermocycler (Applied Biosystems, Foster City, CA).

PCR products were purified using multi-screen, 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 5× sequencing buffer, 5.25 µl of 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 (Ewing et al., 1998). The genomic DNA reference sequence from GenBank (NG_007100.1) and the cDNA one (M65131.1) were used in this study, and 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 the *MUT* gene were annotated. The accession numbers to the submitted sequences are “KC594079–KC594098” and available at <http://www.ncbi.nlm.nih.gov/nucleotide/>, 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 submitted to the ClinVar database www.ncbi.nlm.nih.gov/clinvar and LOVD (Celli et al., 2011) https://grenada.lumc.nl/LOVD2-mendelian_genes/home.php.

MCM structural modelization

The cloning and the sequence analysis of both the human MCM (Ledley et al., 1988); (Jansen et al., 1989) and the MCM from *Propionibacterium shermanii* (Marsh et al., 1989) have revealed the very high

amino acid sequence homology (65% identity) between the mature human enzyme and the α -subunit of the *P. shermanii* enzyme. This allowed the construction of a 3D model that satisfies spatial constraints (Thomä and Leadlay, 1996). The human MCM differs in being α homodimer rather than α , β heterodimers, 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 (Ewing et al., 1998), (Gordon et al., 1998). Molecular modeling simulations were performed with the Modeller 9.11 software (Šali and Blundell, n.d.). The input to the program is an alignment of the target sequence with the related three dimensional structures (α chain of the *P. 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 who 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 until 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 a 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 month affected child with a family history of two dead members probably with the same condition. He presented with severe hyperammonemia (336.6 $\mu\text{mol/l}$, 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, and he presented with delayed mental and physical developments, vomiting, cyanosis, irritability and pyelonephritis. *Patient 6*, with no history of a similar condition, was normal until the age of 1 year and 8 months when he was admitted to the PICU with chest infection, bronchial asthma, generalized tonic convulsion, acidosis, disturbed conscious level and delayed motor and mental developments. *Patient 7* and *patient 8*, also with no history of related conditions, had started their symptoms at the age of 8 months and 10 months, respectively, and they presented with severe acidosis, lethargy and disturbed conscious level.

Patient 9 and her older sister were affected with the neonatal-onset form. They presented with tachypnea, disturbed conscious level, loss of acquired motor and mental developments and then coma. *Patient 10* and his two older affected brothers 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 second brothers.

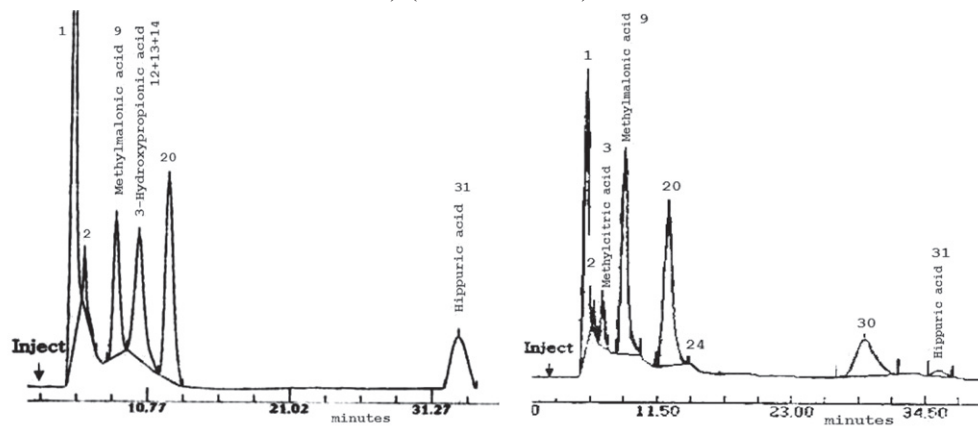
Patient 11 is affected with a neonatal onset-form of propionic acidemia on the 3rd day after birth. Sequencing analysis was performed accidentally when he was suspected with MMA due to elevated C3 (35.9 $\mu\text{mol/l}$) and C3:C2 (0.49) in blood by metabolic screening. He presented with jaundice and a severe hyperammonemia (206 $\mu\text{mol/l}$ [reference range < 48]).

Biochemical investigations

Among MMA patients, routine laboratory tests have reported anemia (60%), severe metabolic acidosis (60%), as well as impaired functions of the liver (20%), kidneys (62.5%), and cardiac muscle (10%). Ammonia level was [163.81 \pm 101.76 $\mu\text{mol/l}$] [mean \pm standard deviation (SD), reference range < 48] indicating that about 54.55% of the patients had hyperammonemia prior to treatment. Mean hemoglobin level was [9.43 \pm 1.75 g/dl], mean pH was [7.34 \pm 0.09], mean anion gap was [15.95 \pm 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.

METHYLMALONIC ACIDURIA (MMA)

A) (KETOACIDOSIS)



B) (ABSENCE OF KETOACIDOSIS)

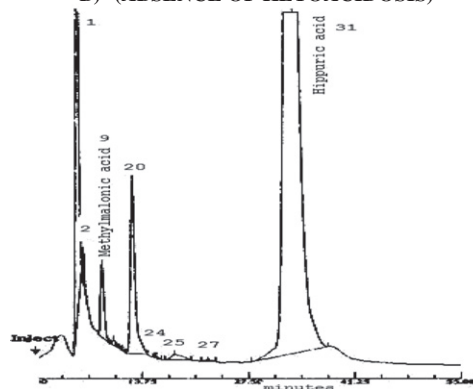


Fig. 2. Urinary organic acids from patients with methylmalonic aciduria. Diagnostic peaks are as follows: 3, methylcitric acid [6.89 ± 0.06]; 9, methylmalonic acid [8.60 ± 0.06]; 12, 3-hydroxypropionic acid [9.98 ± 0.21]. A) Untreated methylmalonic aciduria patients showed highly elevated MMA, methylcitric acid and 3-hydroxypropionic acid. B) Diet controlled MMA patient; an abnormal peak corresponding to hippuric acid can be seen, along with a smaller peak of methylmalonic acid (Ghoraba et al., 2014). Propionic aciduria (PA). A) Ketoacidosis. B) Absence of ketoacidosis.

Metabolic profiling and HPLC urinary organic acid analysis

All MMA patients were diagnosed by elevated levels of propionylcarnitine (C3), ratios of C3/acetylcarnitine (C2) in blood, and increased level of methylmalonic acid in urine. Blood levels of C3 and C3/C2 were $[22.29 \mu\text{mol/l} \pm 11.39]$ and $[3.16 \pm 5.01]$, respectively (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.

Fig. 2A shows different HPLC profiles of MMA patients that show 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 (Fig. 2B) (Ghoraba et al., 2014).

Fig. 3 shows profiles from the propionic acidemia patient before (a) and after (b) management (Ghoraba et al., 2014).

Mutation study

PCR primers were designed for amplification of a 552 bp genomic region (g.8588–g.9132) of the *MUT* gene exon 2 (g.8635–g.9058) (c.-39-385) and involved the following: 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 1i), as well as an 81 bp downstream ORF intron (intron 2i). All PCR amplicons of the *MUT* gene exon 2 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 patient 4, who has not shown any amplification for exon 2 (Fig. 4).

Comparison of DNA sequences obtained for the patients with the consensus sequences of the genomic region of the *MUT* gene (GenBank accession number NG_007100.1:g.8588–g.9132) (Fig. 5) and human MCM c.DNA (GenBank accession number M65131.1 or NG_007100.1:c.-39-385) (Fig. 6) has revealed a total of 26 variants (Table 1).

Patient 1 was homozygous to the only previously reported mutation c.323G>A (p.R108H) (Acquaviva et al., 2001), while the other 25 were novel allelic variants. Three novel mutations were located in the coding region of the *MUT* exon 2: a missense mutation consists of C>A transversion at position 165, c.165C>A (p.N55K), a silent mutation consists of G>A transition at position 15, c.15G>A (p.K5K), these two mutations were identified in more than one patient, 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, and the third novel mutation was the frame shift mutation c.7del (p.R3EfsX14) that identified in patient 10 (Table 2).

Four mutations were involved in the acceptor/donor consensus splice-site sequences: 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. SNPs were dispersed throughout the intronic regions and upstream to the exon 2 coding region as well. Common identified polymorphisms were as follows: 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), while no significant mutations have been detected in the *MUT* exon 2 of family 8 (Fig. 5, Table 1). All 26 allelic variants identified in this study are available at (http://www.ncbi.nlm.nih.gov/SNP/snp_viewBatch.cgi?sbid=1059777) (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.R3EfsX14 in families 1 and 10, respectively, was the clinical severity, but was variable in both patients, whereas neonatal onset was another clinical feature for p.R108H in family 1. The frame shift mutation, p.R3EfsX14, was a distinctive clue for the clinical severity as well as the hepatic dysfunction reported in family 10 (Table 2).

MCM associated p.N55K modelization study

Multiple sequence alignment of MCM amino acid sequence around Asn55 in various species: (*Homo sapiens*, *P. shermanii*, *Mus musculus*, *Escherichia coli*, *Mycobacterium tuberculosis*, and *Caenorhabditis elegans*

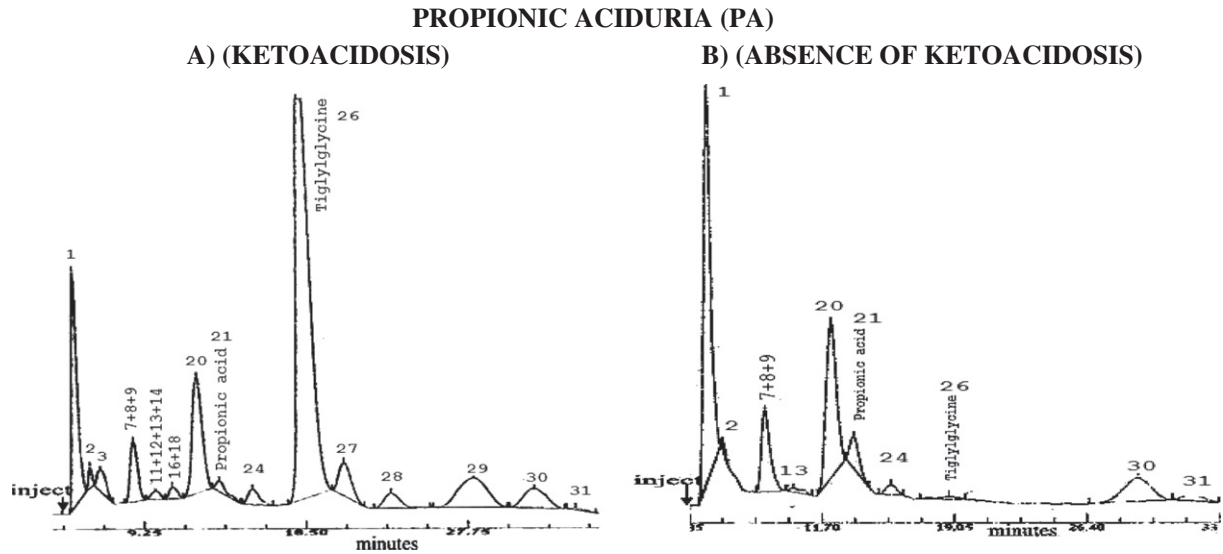


Fig. 3. Urinary organic acids from a patient with propionic aciduria. Diagnostic peaks are as follows: 3, methylcitric acid [6.89 ± 0.06]; 8, propionylglycine [8.58 ± 0.01]; 12, 3-hydroxypropionic acid [9.98 ± 0.21]; 13, lactic acid [10.19 ± 0.09]; 14, 3-hydroxybutyric acid [10.32 ± 0.10]; 16, 3-hydroxyisovaleric acid [10.63 ± 0.04]; 18, 2-methylacetoacetic acid [10.85 ± 0.06]; 21, propionic acid [13.37 ± 0.26]; 26, tiglylglycine [18.49 ± 0.49] (Ghoraba et al., 2014).

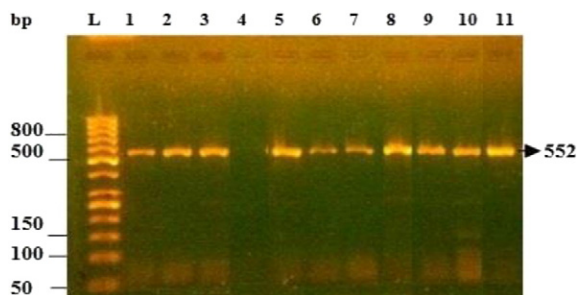


Fig. 4. Agarose gel electrophoresis of PCR products of the patient samples. (Left) lane L, 50 bp DNA ladder (Invitrogen, USA), Lanes from 1 to 11 are PCR products of MUT exon 2 for 11 patients resulting in a remarkable 552 bp DNA fragment in all patient samples except for patient 4.

(Fig. 7)), indicates that Asn55 has low conservative level and is only conserved in humans and mice. Mapping of the p.N55K substitution on the three dimensional model of human MCM (Thomä and Leadlay, 1996) shows that the Asn-55 residue lies in the extreme N-terminus of methylmalonyl-CoA mutase and does not contribute to neither binding of substrate nor the active site (Fig. 8).

Discussion

Here, we report the results of mutation analysis of the *MUT* gene exon 2 in eleven unrelated Egyptian families, and we highlight some important aspects of methylmalonic aciduria diagnosis. 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. Routine laboratory tests have commonly reported hyperammonemia, anemia and severe metabolic acidosis, as well as impaired functions of the liver, kidneys, and cardiac muscle. Methylmalonic acid in urine was easily detected by isocratic cation exchange high performance liquid chromatography (HPLC) in the initial attacks of MMA where methylmalonic, methylcitric and 3-hydroxypropionic acids were significantly elevated in urine for ten patients (1–10). This method has been used routinely in our department for initial screening of urine samples from neonates and infants suspected of having organic aciduria (Ghoraba et al., 2014), but confirmation analysis by using GC/MS would still be needed (Han et al., 2007). 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; whilst, *patient 11* has been included in the mutation study before the final diagnosis with propionic acidemia by using GC/MS.

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 (Hörster and Hoffmann, 2004), (Nicolaidis et al., 1998). In about eight patients, MMA decreased consistently after treatment; they even returned to normal levels; these approaches match those reported by Hörster and Hoffmann (2004).

In the present study, the genomic region (g.8588–g.9132) of the *MUT* gene exon 2 (g.8588–g.9058) (c.-39–385), including a 385 bp coding region (g.8674–g.9058) that 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), was amplified and then sequenced to all studied patients, including *patient 11*, as an approach to report common mutations of the *MUT* gene exon 2 within Egyptian patients. The findings of PCR products matched those reported by Worgan et al. (Jung et al., 2005) since a 552 bp DNA fragment was detected in all patients except *patient 4* who has not shown a PCR product for exon 2. This is probably expected due to exon 2 deletion; therefore it would be important to analyze the other *MUT* exons for this patient.

Former studies have reported various mutations within exon 2 of the *MUT* gene (Acquaviva et al., 2005; Cavicchi et al., 2001; Heptinstall et al., 1999; Jung et al., 2005; Ledley and Rosenblatt, 1997; Martínez et al., 2005; Mikami et al., 1999); (Acquaviva et al., 2001). However, bi-directional gDNA sequencing for all PCR amplicons in the recent study has revealed a total of 26 variants. The only previously reported mutation was the homozygous mutation c.323G>A (p.R108H) in *patient 1* which was reported in white and Korean

8588	<u>T CCCACCCCT</u>	<u>CTTCTAAATG</u>	TTTTACTCT	ATGTTTCTTT	TTCTAGGTCA	8638
				G ▲	A +A ³ A ²	
8639	GTTCTTATTT	CTATTGGGTG	TTTCCATGCT	CCACC <u>ATGTT</u>	AAGAGCTAAG	8688
	TA+A	A	A A A ²		▲ A ³	
8689	AATCAGCTTT	TTTTACTTTC	ACCTCATTAC	CTGAGGCAGG	TAAAAGAATC	8738
8739	ATCAGGCTCC	AGGCTCATAC	AGCAACGACT	TCTACACCAG	CAACAGCCCC	8788
8789	TTCACCCAGA	ATGGGCTGCC	CTGGCTAAAA	AGCAGCTGAA	AGGCAAAAAC	8838
					A ³	
8839	CCAGAAGACC	TAATATGGCA	CACCCGGAA	GGGATCTCTA	TAAAACCCTT	8888
8889	GTATTCCAAG	AGAGATACTA	TGGACTTACC	TGAAGAACTT	CCAGGAGTGA	8938
8939	AGCCATTCAC	ACGTGGACCA	TATCCTACCA	TGTATACCTT	TAGGCCCTGG	8988
8989	ACCATCCGCC	AGTATGCTGG	TTTTAGTACT	GTGGAAGAAA	GCAATAAGTT	9038
	A					
9039	CTATAAGGAC	AACATTA <u>AGG</u>	GTGAGATTTT	AATGTAAGAC	ATAATATTTA	9088
			C ²	+T	▲ ² C	
9089	TGATAAGTCA	TATGCCTCCT	AGTTTTTTAT	<u>TTTTTGGGGG</u>	<u>TTAATCTCTG T</u>	9139
	▲ C ² +C	▲ ▲				

Fig. 5. Sequence alignment of exon 2 of human methylmalonyl CoA mutase in the nucleotide level indicating the position of identified individual mutations with their recurrent number printed above the mutation; positions of forward and reverse primers are indicated in underlined bold, while the coding region lies between the dark gray AUG starting codon and the AAG codon which codes for the 128th amino acid residue (Lys).

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1          10          20          30          40          50          59
H sapiens MLRAKNQLFLLSPHYLRQVKESSSGSRLIQQRLLHQQQPLHPEWAALAKKQLKGNPEDL
MCM1      MLRAKNQLFLLSPHYLRQVKESSSGSRLIQQRLLHQQQPLHPEWAALAKKQLKGNPEDL
MCM2      MLRAKNQLFLLSPHYLRQVKESSSGSRLIQQRLLHQQQPLHPEWAALAKKQLKGNPEDL
MCM3      MLRAKNQLFLLSPHYLRQVKESSSGSRLIQQRLLHQQQPLHPEWAALAKKQLKGNPEDL
MCM5      MLRAKNQLFLLSPHYLRQVKESSSGSRLIQQRLLHQQQPLHPEWAALAKKQLKGNPEDL
MCM6      MLRAKNQLFLLSPHYLRQVKESSSGSRLIQQRLLHQQQPLHPEWAALAKKQLKGNPEDL
MCM7      MLRAKNQLFLLSPHYLRQVKESSSGSRLIQQRLLHQQQPLHPEWAALAKKQLKGNPEDL
MCM8      MLRAKNQLFLLSPHYLRQVKESSSGSRLIQQRLLHQQQPLHPEWAALAKKQLKGNPEDL
MCM9      MLRAKNQLFLLSPHYLRQVKESSSGSRLIQQRLLHQQQPLHPEWAALAKKQLKGNPEDL
MCM10     MLELRISSEFYEHLLIIGRKNHQAPGSSYNDFEYISNSPETQNGLPWLKSSSKAKTIQKI
MCM11     MLRAKNQLFLLSPHYLRQVKESSSGSRLIQQRLLHQQQPLHPEWAALAKKQLKGNPEDL

60         70         80         90         100        110        119
H sapiens I WHTPEGISIKPLYSKRDTMDLPEELPGVKPFTTRGPYP TMYTFRPWTIRQYAGFSTVEES
MCM1      I WHTPEGISIKPLYSKRDTMDLPEELPGVKPFTTRGPYP TMYTFRPWTIRQYAGFSTVEES
MCM2      I WHTPEGISIKPLYSKRDTMDLPEELPGVKPFTTRGPYP TMYTFRPWTIRQYAGFSTVEES
MCM3      I WHTPEGISIKPLYSKRDTMDLPEELPGVKPFTTRGPYP TMYTFRPWTIRQYAGFSTVEES
MCM5      I WHTPEGISIKPLYSKRDTMDLPEELPGVKPFTTRGPYP TMYTFRPWTIRQYAGFSTVEES
MCM6      I WHTPEGISIKPLYSKRDTMDLPEELPGVKPFTTRGPYP TMYTFRPWTIRQYAGFSTVEES
MCM7      I WHTPEGISIKPLYSKRDTMDLPEELPGVKPFTTRGPYP TMYTFRPWTIRQYAGFSTVEES
MCM8      I WHTPEGISIKPLYSKRDTMDLPEELPGVKPFTTRGPYP TMYTFRPWTIRQYAGFSTVEES
MCM9      I WHTPEGISIKPLYSKRDTMDLPEELPGVKPFTTRGPYP TMYTFRPWTIRQYAGFSTVEES
MCM10     YGTPRKGSLLNPCI PREILWIYLNKEQESSHSHVDHILPCIPLGPGPSASMLVVLVWKKKA
MCM11     I WHTPEGISIKPLYSKRDTMDLPEELPGVKPFTTRGPYP TMYTFRPWTIRQYAGFSTVEES

120        128
H sapiens NKFYKDNIK
MCM1      NKFYKDNIK
MCM2      NKFYKDNIK
MCM3      NKFYKDNIK
MCM5      NKFYKDNIK
MCM6      NKFYKDNIK
MCM7      NKFYKDNIK
MCM8      NKFYKDNIK
MCM9      NKFYKDNIK
MCM10     ISSIRITLX
MCM11     NKFYKDNIK

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Fig. 6. Partial protein alignment of the amino acid residues (1–128) of MCM for the studied patients; positions of individual mutations are indicated in underlined bold.

Table 1Results of the mutation study of the *MUT* gene exon 2 in 10 Egyptian families with MMA and one Egyptian patient with PA.

ID	Age at onset	Diagnosis	Sex	Nucleotide change		Hom/Het
				DNA	c.DNA	
1	4 months	MMA	M	g.8657T>A	c.-17T>A	Het
				g.8663C>A	c.-11C>A	Het
				g.8668T>A	c.-6T>A	Het
				g.8688G>A	c.15G>A	Hom
				g.8838C>A	c.165C>A	Het
				g.8996G>A	c.323G>A	Hom
2	12 months	MMA	F	g.9067T>C	c.385 + 9T>C	Het
				g.8622T>G	c.-39-13T>G	Het
				g. 8634_8635insA ^a	c.-39-1_-39insA	Het
3	15 months	MMA	F	g.9104delC	c.385 + 46delC	Hom
				g. 8634_8635insA ^a	c.-39-1_-39insA	Het
5	3 days	MMA	M	g.8637C>A	c.-37C>A	Het
				g.8639G>T	c.-35G>T	Hom
				g.8640T>A	c.-34T>A	Het
				g.8640_8641insA	c.-34_-33insA	Het
				g.9087delT	c.385 + 29delT	Hom
				g.9091A>C	c.385 + 33A>C	Hom
				g.9092_9093insC	c.385 + 34_385 + 35insC	Hom
				g.8632T>A ^a	c.-39-3T>A	Het
6	20 months	MMA	M	g.8688G>A	c.15G>A	Het
				g.9088A>C	c.385 + 30A>C	Het
				g.9089delT	c.385 + 30A>C	Het
7	8 months	MMA	F	g.8626delT*	c.-39-9delT	Het
				g.8632delT*	c.-39-3delT	Het
9	6 days	MMA	F	g.8634_8635insA ^a	c.-39-1_-39insA	Het
				g.8637C>A	c.-37C>A	Het
				g.9087delT	c.385 + 29delT	Het
				g.9091A>C	c.385 + 33A>C	Het
				g.9101delT	c.385 + 43delT	Het
10	NR	MMA	M	g.8838C>A	c.165C>A	Het
				g.8680delA	c.7delA	Hom
11	3 days	PA	M	g.9067T>C	c.385 + 9T>C	Hom
				g.8661T>A	c.-13T>A	Het
				g.8668T>A	c.-6T>A	Het
				g.8688G>A	c.15G>A	Het
				g.8838C>A	c.165C>A	Het
				g.9076_9077insT	c.385 + 18_385 + 19insT	Het

MMA; methylmalonic aciduria, PA; propionic aciduria, M; male, F; female, NR; not recorded, Hom; homozygous, Het; heterozygous.

The genomic DNA reference sequence from GenBank is NG_007100.1 and the cDNA reference sequence one is M65131.1.

Patient 4 has not recorded exon 2 amplification, while patient 8 has not detected any significant mutation.

^a Mutations predicted to affect splicing.

patients (Acquaviva et al., 2001). The highly conserved arginine at position 108 is in the first β -sheet of the N-terminal ($\beta\alpha$)₈ barrel and is directly involved in binding the ADP-ribosyl moiety of the CoA ester substrate at the entrance of the substrate channel (Thomä and Leadlay, 1996). 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 (Worgan et al., 2006) and p.E117X in Japanese patients (Ogasawara et al., 1994). Likewise, this study has revealed that two heterozygous novel mutations were likely to be recurrent rather than inherited from a common ancestor and were suggested to be common within the Egyptian population. First, the heterozygous silent mutation c.15G>A (p.K5K), identified in families (1, 5 and 11), is located in the mitochondrial leader sequence. It has a silent effect on the transcribed amino acid (Lys residue), MCM folding as well as the whole MCM enzymatic activity; therefore c.15G>A (p.K5K) was suggested to be a common natural polymorphism. Second, is the heterozygous missense mutation c.165C>A (p.N55K) identified in families (1, 9 and 11). Homology model of c.165C>A (p.N55K) mutation of human MCM constructed by Modeller 9.11 on the basis

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

ID	Gender	Diagnosis	Age at onset	Presenting symptoms	C3	C3/C2	Mutation		Variant remarks	Hom/Het	Dom
							Nucleotide	Amino acid			
1	M	MMA	4 months	Delayed motor and mental developments, lethargy, tachypnea, metabolic acidosis, hyperammonemia, vomiting, fever, anemia and diarrhea	30.11	0.47	c.15G>A c.165C>A c.323G>A	p.K5K ^a p.N55K ^a p.R108H ^b	Silent Missense Missense	Hom Het Hom	ML NT (β α) ₈
5	M	MMA	3 days	Delayed motor and mental developments, lethargy, and bad obstetric history	11.4	0.7	c.15G>A	p.K5K	Silent	Het	ML
9	F	MMA	6 days	Tachypnea, disturbed conscious level then coma, loss of acquired motor and mental developments, lethargy, hyperammonemia, anemia and PICU admission	26.3	0.67	c.165C>A	p.N55K	Missense	Het	NT
10	M	MMA	NR	Enlarged liver, otitis media, tonsillitis, fever, developmental regression, loss of motor milestone, vomiting, metabolic acidosis and coma	NR	NR	c.7del	p.R3EfsX14 ^a	Frame shift	Hom	ML
11	M	PA	3 days	Hyperammonemia, jaundice, anemia and NICU admission	35.9	0.49	c.15G>A c.165C>A	p.K5K p.N55K	Silent Missense	Het Het	ML NT

M; male, F; female, MMA; methylmalonic aciduria, PA; propionic aciduria, PICU; pediatric intensive care unit, NICU; neonatal intensive care unit, NR; not recorded, Hom; homozygous, Het; heterozygous, C3: propionylcarnitine, C3:C2; acetylcarnitine:propionylcarnitine, Dom; domain, ML; mitochondrial leader sequence, NT; N-terminal extended segment, (β α)₈; N-terminal barrel.

Normal reference values; C3<4.0 μmol/l, C3:C2<0.30.

^a Novel mutations.

^b Mutation involves CpG dinucleotide.

Table 3

ClinVar submission summary of 26 SNPs in the MUT gene exon 2.

Total SNPs submitted:		26		
dbSNP batch ID:		1059777		
HGVS name	Number of independent observations	GenBank sequence ID	subSNP_ID	SNP_ID
1 NG_007100.1:g.8996G>A	1	KC594079.1; KC594080.1	ss995476987	rs483352778
2 NG_007100.1:g.8688G>A	3	KC594079.1; KC594085.1; KC594096.1	ss995476988	rs483352779
3 NG_007100.1:g.8838C>A	3	KC594079.1; KC594092.1; KC594096.1	ss995476989	rs483352780
4 NG_007100.1:g.8680delA	1	KC594094.1	ss995476990	rs483352781
5 NG_007100.1:g.8668T>A	2	KC594079.1; KC594096.1	ss995476991	rs483352782
6 NG_007100.1:g.9067T>C	2	KC594080.1; KC594095.1	ss995476992	rs483352783
7 NG_007100.1:g.8661T>A	1	KC594096.1	ss995476993	rs483352784
8 NG_007100.1:g.9088A >	1	KC594086.1	ss995476994	rs483352785
9 NG_007100.1:g.9091A>C	2	KC594084.1; KC594090.1	ss995476995	rs483352786
10 NG_007100.1:g.9087delT	2	KC594084.1; KC594090.1	ss995476996	rs483352787
11 NG_007100.1:g.9089delT	1	KC594086.1	ss995476997	rs483352788
12 NG_007100.1:g.8657T>A	1	KC594079.1	ss995476998	rs483352789
13 NG_007100.1:g.8663C>A	1	KC594079.1	ss995476999	rs483352790
14 NG_007100.1:g.8622T>G	1	KC594081.1	ss995477000	rs483352791
15 NG_007100.1:g.8634_8635insA	3	KC594081.1; KC594083.1; KC594089.1	ss995477001	rs483352792
16 NG_007100.1:g.9104delC	1	KC594082.1	ss995477002	rs483352793
17 NG_007100.1:g.8637C>A	2	KC594083.1; KC594089.1	ss995477003	rs483352794
18 NG_007100.1:g.8639G>T	1	KC594083.1	ss995477004	rs483352795
19 NG_007100.1:g.8640T>A	1	KC594083.1	ss995477005	rs483352796
20 NG_007100.1:g.8640_8641insA	1	KC594083.1	ss995477006	rs483352797
21 NG_007100.1:g.9092_9093insC	1	KC594084.1	ss995477007	rs483352798
22 NG_007100.1:g.8632T>A	1	KC594085.1	ss995477008	rs483352799
23 NG_007100.1:g.8626delT	1	KC594087.1	ss995477009	rs483352800
24 NG_007100.1:g.8632delT	1	KC594087.1	ss995477010	rs483352801
25 NG_007100.1:g.9101delT	1	KC594090.1	ss995477011	rs483352802
26 NG_007100.1:g.9076_9077insT	1	KC594097.1	ss995477012	rs483352803

of homology with the *P. shermanii* enzyme (Thomä and Leadlay, 1996), (Acquaviva et al., 2001) has shown that the N55K mutation is located in the extreme N-terminal. It also indicated that the missense mutation c. 165C>A (p.N55K) may affect the overall MCM conformation in three different aspects. First, 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 homodimerization and formation of heterodimers. Second, the increased size of the side chain is likely to lead to unfavorable folding. Third, the introduction of the hydrophobic Lys residue on the surface of the domain is energetically unfavorable and leads to unfavorable charge–charge interaction, which might hamper the positioning of adjacent helix in the MCM homodimers (due to steric clash). Thus, the hydrophobic Lys side chain would disrupt the favorable interactions, leading to change in N-terminal folding that may interfere with the homo dimerization necessary for MCM activity. On the other hand, the low conservative level of the Asn 55 residue in various species (*H. sapiens*, *P. shermanii*, *M. musculus*, *E. coli*, *M. tuberculosis*, and *C. elegans*), the heterozygosity of the mutation and its recurrence in *patient 11* with propionic acidemia make the c.165C>A (P.N55K) mutation very likely to be pathogenically insignificant and do not interfere the MCM enzymatic catalysis in studied patients. Therefore, c.165C>A (p.N55K) is expected to be a frequent heterozygous mutation within the Egyptian population.

Moreover, a third novel frame shift mutation c.7del (p.Arg3GlufsX14) identified in *patient 10* was located in the *MUT* exon 2 coding region. It is located in the mitochondrial leader sequence which results in major amino acid changes and subsequent premature stop codons.

Four mutations located in the acceptor/donor consensus splice-site sequences and predicted to affect splicing were identified in this study: c.-6T>A (in families 1 and 11), c.385+9T>C (in families 1 and 10),

										↓
1	MLRAKNQFLFLSPHYLRQVKES	SGSRLIQORLLHQOQPLHPEWAALAKKOLK	--GKNPED	58	P22033	MUTA_HUMAN				
1	-----MSTLPR	----FDSVDLGN	---AFVPADAARRFEELAA-----KAGTG	35	P11653	MUTB_PROFR				
1	MLRAKNQFLFLSPHYLKQLNI	PSASRW--KRLHQQOPLHPEWAFLAKKOLK	--GKNPED	56	P16332	MUTA_MOUSE				
1	-----	-----MSNVOEWOLANKELSRR	REKTIVDS	24	P27253	SCPA_ECOLI				
1	-----MTTKIFVIGS	----FAGVPLHSERAAQSPT	EAAVHthVAaaaaah--GYTPEQ	47	P71774	MUTB_MYCTU				
1	----MYLQLLKPTLLRCSTRPSS	-----GAYTRS	PIDQWAAMAKKAMK--GREADT	47	Q23381	MUTA_CAEEL				

Fig. 7. Partial alignment of MCM amino acid sequence around Asn55 in *Homo sapiens*, *Propionibacterium shermanii*, *Mus musculus*, *Escherichia coli*, *Mycobacterium tuberculosis* and *Caenorhabditis elegans*, with 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 the Asn-55 residue which is conserved in humans and in mice. Resource is available at <http://www.uniprot.org/align/20130524404TMUYT7Q>.

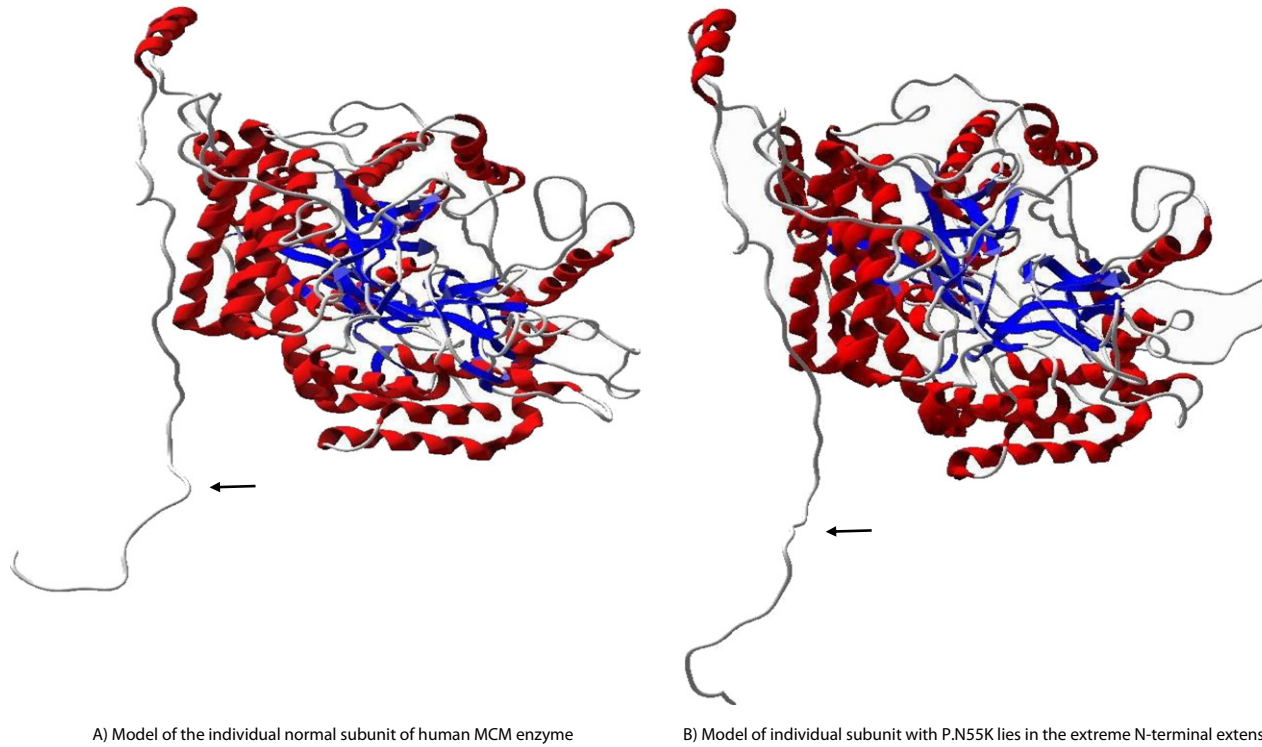


Fig. 8. View of the three-dimensional structure of the human methylmalonyl-CoA mutase enzyme models built on the basis of experimental structure of the α chain of the *P. shermanii* enzyme (PDB: 1REQ) and the human MCM enzyme (PDB: 3BIC and 2XIQ) by Modeller 9.11, showing an increased size and steric clash made by the hydrophobic positively charged Lys residue located at position 165 in the extreme N-terminal extension. A) Model of the individual normal subunit of the human MCM enzyme. B) Model of individual subunit with P.N55K lies in the extreme N-terminal extension.

c.-37C>A, c.385 + 29delT and c.385 + 33A>C (in families 3 and 7). However, restriction analysis and mutation studies to the other *mut* exons would provide a valuable confirmation to the pathogenicity of identified mutations and reveal the phenotype–genotype correlations.

Single nucleotide polymorphisms were spread all over the intronic non-coding areas of the MUT gene exon 2 and were reported within all families except family 8 who has not reported any significant mutations.

To conclude, the current study has identified 25 novel allelic variants: ten of which were intronic, eight were located upstream to the exon 2 coding region, four were novel modifications predicted to affect splicing and three were novel mutations within the coding region (c.15G>A, c.165C>A and c.7del), as well as a previously reported mutation c.323G>A was identified. Genetic heterozygosity was high among the identified mutations; therefore the phenotype resulting from compound heterozygosity has not been precisely characterized. The two identified heterozygous mutations, c.15G>A and c.165C>A, were suggested to be common within Egyptian families. However, 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 were identified in family 8. Meanwhile, it would be important to analyze the other MUT exons as well as the 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.

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