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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 B_{12}) 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 (propionylcarnitine) and C3:C2 (propionylcarnitine/acetylcarnitine) 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.K5K), 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 methylmalonyl 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 methylmalonic acid metabolism (termed *mut* MMA or vitamin B_{12}-unresponsive MMA) [1], or in genes required for the metabolism of its cofactor, 5'-deoxyadenosylcobalamin (AdoCbl) (called cbl MMA or vitamin B_{12}-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].

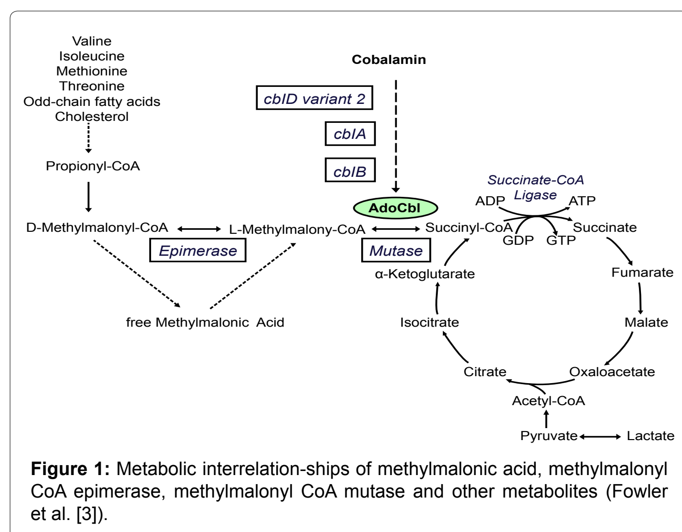

Figure 1: Metabolic interrelation-ships of methylmalonic acid, methylmalonyl CoA epimerase, methylmalonyl CoA mutase and other metabolites (Fowler et al. [3]).

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]. MCM is encoded by *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 [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 $(\beta\alpha)_8$ 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).

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 hydroxylcobalamin 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].

Various studies have identified different disease-causing mutations in human *MUT* gene [16-22]. Different studies reported mutations specific populations, including c.322C>T (p.R108C) in Hispanic patients, c.1630–1631delGGinsTA (p.G544X) and c.1280G>A (p.G427D) in Asian patients [23], p.G717V in black patients [24], p.E117X in Japanese patients [25], c.655A4T (p.N219Y) in Caucasian patients [26], c.1595G>A, c.2011A>G in Filipino patients [27], 1048delT and 1706_1707delGGinsTA (p.G544X) in Thai patients [28], and the c.671-678dup in Spanish patients [21].

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 the majority of 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 MMA, 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 of having *mut* 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, 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 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 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 >5min, 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 Helsiniki) 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., QP2010). 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-g9132) of *MUT* gene (NG_007100.1) exon 2 (g.8635-g.9058) (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 1i), 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) $MgCl_2$ (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 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	Reverse primer
5'-TCCCACCCCCTCTTCTAAAT-3'	5'-ACAGAGATTAACCCCCAAAAA-3'

[a]Reported previously by (Worgan et al. [23])

Table 1: Exon 2 primers sequences[a].

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 *MUT gene* were annotated. The accession numbers to the submitted sequences are "KC594079-KC594098" 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 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. 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 dimentional 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 convulsion, 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].

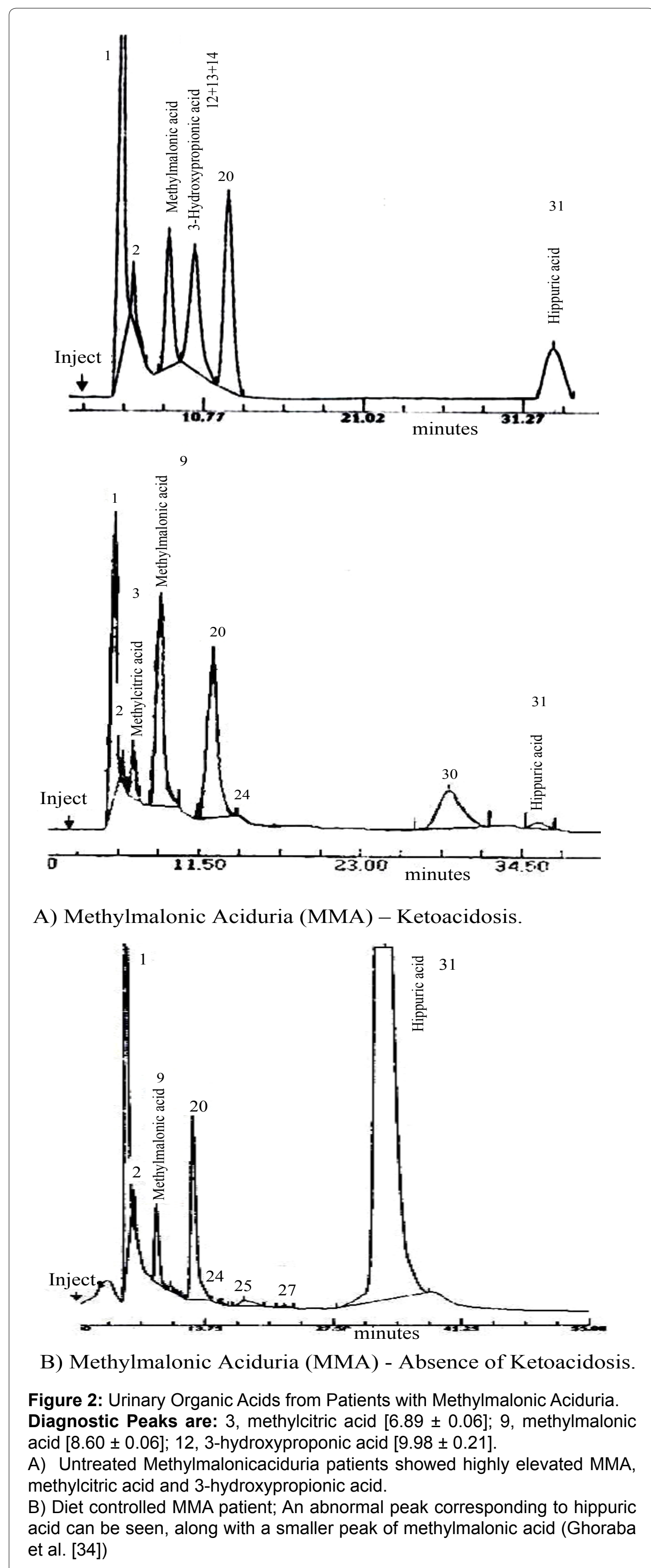
Biochemical investigations

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/acetylcarnitine (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



A) Methylmalonic Aciduria (MMA) – Ketoacidosis.

B) Methylmalonic Aciduria (MMA) - Absence of Ketoacidosis.

Figure 2: Urinary Organic Acids from Patients with Methylmalonic Aciduria.
Diagnostic Peaks are: 3, methylcitric acid [6.89 ± 0.06]; 9, methylmalonic acid [8.60 ± 0.06]; 12, 3-hydroxyproponic acid [9.98 ± 0.21].
A) Untreated Methylmalonicaciduria 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. [34])

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.

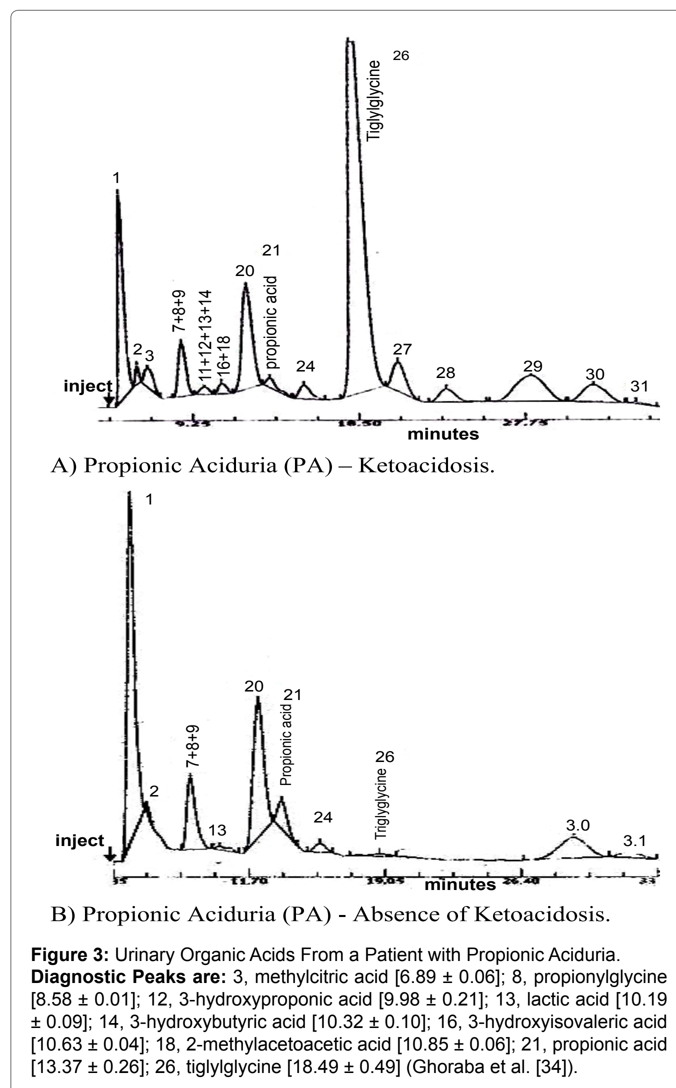
Mutation study

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



A) Propionic Aciduria (PA) – Ketoacidosis.

B) Propionic Aciduria (PA) - Absence of Ketoacidosis.

Figure 3: Urinary Organic Acids From a Patient with Propionic Aciduria.
Diagnostic Peaks are: 3, methylcitric acid [6.89 ± 0.06]; 8, propionylglycine [8.58 ± 0.01]; 12, 3-hydroxyproponic 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. [34]).

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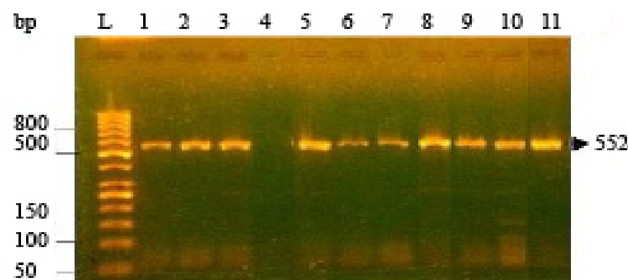

(left) Lane L, 50bp DNA ladder (Introgen, USA), Lanes from 1 to 11 are PCR products of MUT exon 2 for 11 patients resulting in a remarkable 552bp DNA fragment in all patient samples except for patient 4.

Figure 4: Agarose gel electrophoresis of PCR products of the patient samples.

8588	**T CCCACCCCCT**	**CTTCTAAATG**	TTTTTACTCT	ATGTTTCTTT	TTCTAGGTCA	8638
				G ▲	A +A^3 A^2 ▲	
8639	GTTCTTATTT	CTATTGGGTG	TTTCCATGCT	CCACC**ATG**TT	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	CACCCCGGAA	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**AGG**	GTGAGATTTT	AATGTAAGAC	ATAATATTTA	9088
			C^2	+T	▲^{2}C	
9089	TGATAAGTCA	TATGCCTCCT	AGTTTTTTA**T**	**TTTTTGGGGG**	**TTAATCTCTG T**	9139
	▲ C^2 +C	▲ ▲				

Figure 5: Sequence alignment of exon 2 of human methylmalonyl CoA mutase in the nucleotide level indicating 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 coding region lies between the dark gray AUG starting codon and AAG codon which codes for the 128th amino acid residue (Lys).

(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 andc.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.R3EfsX14 in families 1 and 10 respectively, was the clinical severity, but also was variable in both

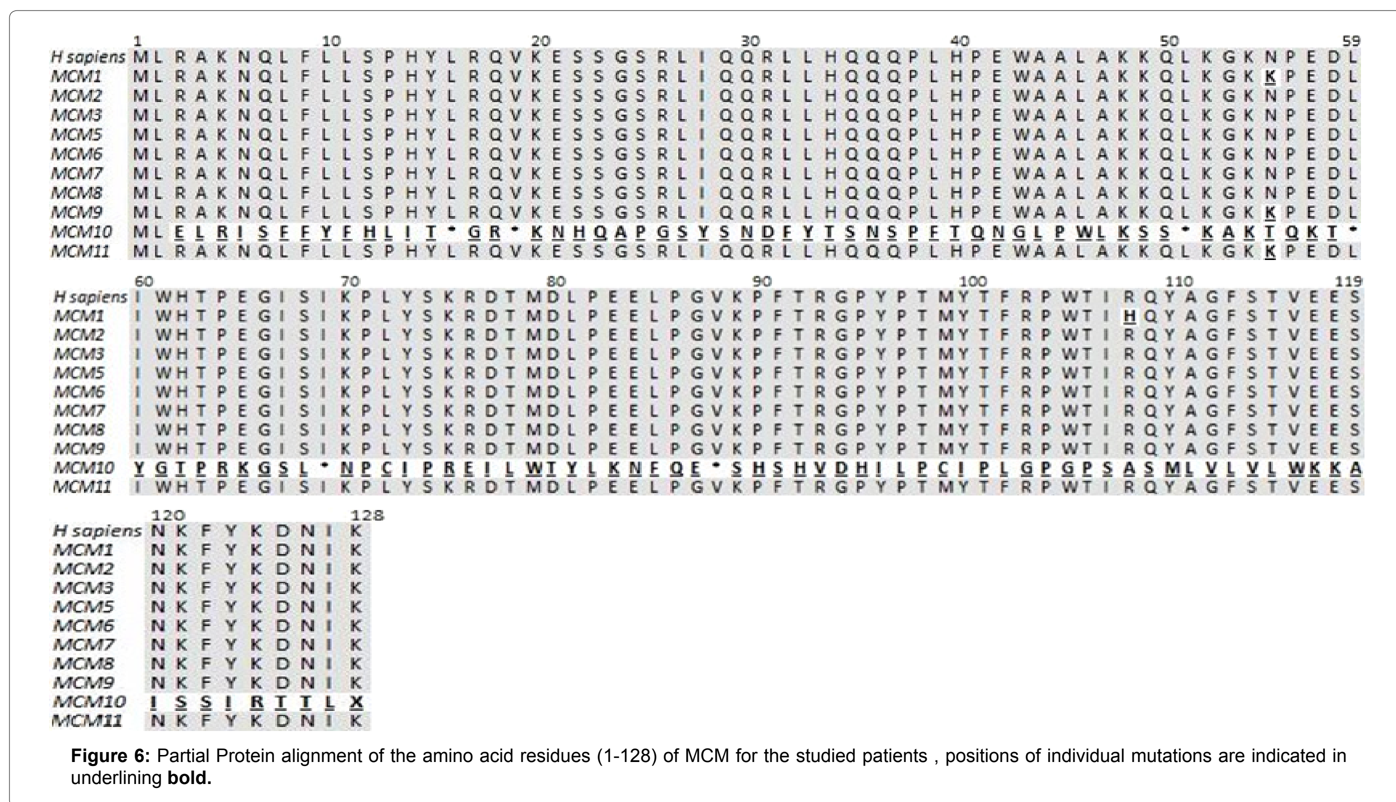

Figure 6: Partial Protein alignment of the amino acid residues (1-128) of MCM for the studied patients , positions of individual mutations are indicated in underlining **bold.**

ID	Gender	Diagnosis	Age of Onset	Presenting Symptoms	C3	C3/C2	Mutation		Variant Remarks	Hom. / Het.	Dom.
							Nucleotide	Amino acid			
1	**M**	MMA	**4 months**	Delayed motor and mental development, lethargy, tachypnea, metabolic acidosis, hyperammonemia, vomiting, fever, anemia and diarrhea	30.11	0.47	c.15G>A	p.K5K[a]	Silent	Hom	ML
							c.165C>A	p.N55K[a]	Missense	Het	NT
							c.323G>A	**p.R108H[b]**	**Missense**	**Hom**	**$(\beta\alpha)_8$**
5	**M**	MMA	**3 Days**	Delayed motor and mental development, lethargy, 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 development, lethargy, hyperammonemia, anemia and admitted into PICU.	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	p.K5K	Silent	Het	ML
							c.165C>A	p.N55K	Missense	Het	NT

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

[a]Novel mutations

[b]Mutation involves CpG dinucleotide

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

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.

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

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ID	Onset Age	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
				g.9067T>C	c.385+9T>C	Het
2	12 months	MMA	F	g.8622T>G	c.-39-13T>G	Het
				g. 8634_8635insA[a]	c.-39-1_-39insA	Het
				g.9104delC	c.385+46delC	Hom
3	15 months	MMA	F	g. 8634_8635insA[a]	c.-39-1_-39insA	Het
				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
5	3 Days	MMA	M	g.8632T>A[a]	c.-39-3T>A	Het
				g.8688G>A	c.15G>A	Het
				g.9088A>C	c.385+30A>C	Het
				g.9089delT	c.385+30A>C	Het
6	20 months	MMA	M	g.8626delT*	c.-39-9delT	Het
				g.8632delT*	c.-39-3delT	Het
7	8 months	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
8	6 Days	MMA	F	g.8838C>A	c.165C>A	Het
9	NR	MMA	M	g.8680delA	c.7delA	Hom
				g.9067T>C	c.385+9T>C	Hom
10	3 Days	PA	M	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

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

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 within Egyptian population. The mutation c.7del was identified in the mitochondrial leader sequence which results in conformational protein change and subsequent premature stop codon.

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, methylcitric 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) (c.-39-385), 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 552bp 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.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.K5K) 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).

Compared with the various mutations in exon 2 reported by many

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1   MLRAKNQLFLLSPHYLRQVKESSGSRLIQQRLLHQQQPLHPEWAALAKKQLK--GKNPED   58   P22033   MUTA_HUMAN
1   -----------MSTLPR----FDSVDLGN---APVPADAARRFEELAA-------KAGTG   35   P11653   MUTB_PROFR
1   MLRAKNQLFLLSPHYLKQLNIPSASRW--KRLLHQQQPLHPEWAVLAKKQLK--GKNPED   56   P16332   MUTA_MOUSE
1   ------------------------------------MSNVQEWQQLANKELSRREKTVDS   24   P27253   SCPA_ECOLI
1   -------MTTKTPVIGS----FAGVPLHSERAAQSPTEAAVHTHVAAAAAAH--GYTPEQ   47   P71774   MUTB_MYCTU
1   -----MYLQLLKPTLLRCSTRPSSS------GAYTRSPIDQKWAAMAKKAMK--GREADT   47   Q23381   MUTA_CAEEL
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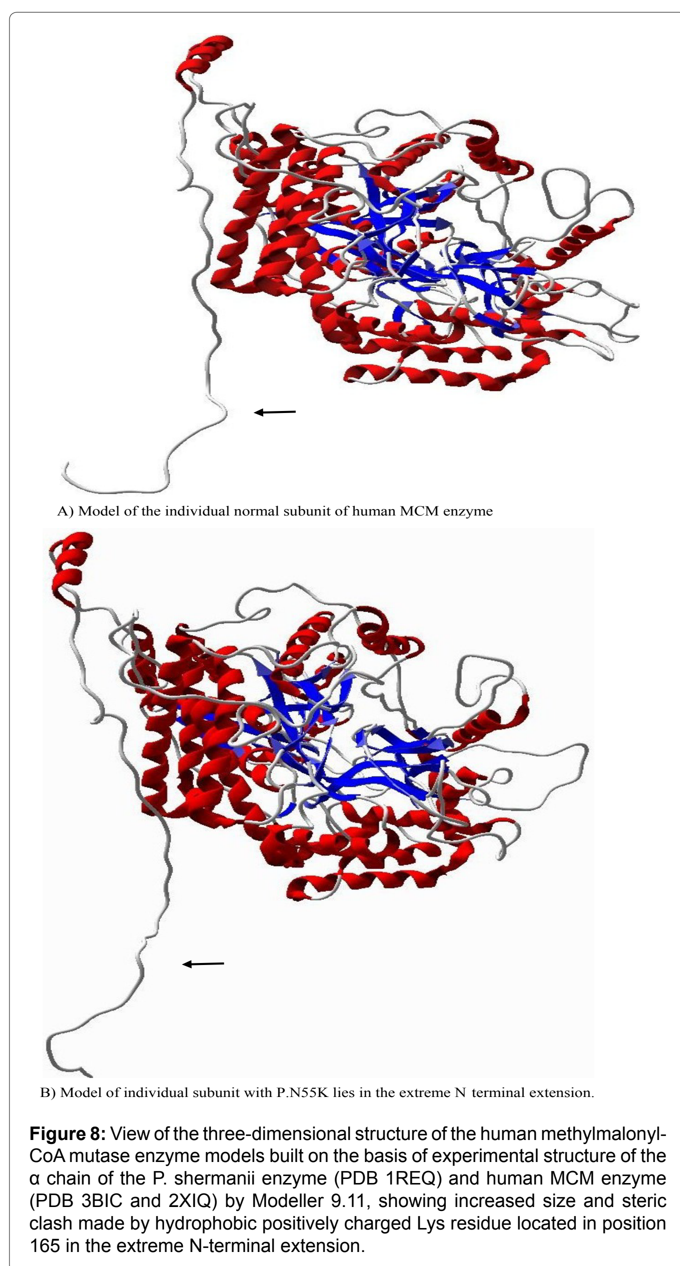
Figure 7: Partial alignment of MCM amino acid sequence around Asn55 is in *Homo sapiens, Propionibacterium shermanii, Mus musculus, Escherichia coli, Mycobacterium tuberculosis and Caenorhabditis elegans.* (Swiss Prot accession numbers P22033, P11653, P16332, P27253, P71774.1 and Q23381 respectively), 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.



A) Model of the individual normal subunit of human MCM enzyme

B) Model of individual subunit with P.N55K lies in the extreme N terminal extension.

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 $(\beta\alpha)_8$ barrel and is directly involved in binding the ADP-ribosyl moiety of the CoA ester substrate at the entrance of the substrate channel [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 (P.N55K) 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 andc.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 heterozyosity 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.

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