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RESEARCH REPORT

Spectrum of Mutations in 60 Saudi Patients with Mut Methylmalonic Acidemia

Faiqa Imtiaz · Bashayer M. Al-Mubarak · Abeer Al-Mostafa · Mohamed Al-Hamed · Rabab Allam · Zuhair Al-Hassnan · Mohammed Al-Owain · Hamad Al-Zaidan · Zuhair Rahbeeni · Alya Qari · Eissa Ali Faqeih · Ali Alasmari · Fuad Al-Mutairi · Majid Alfadhel · Wafaa M. Eyaid · Mohamed S. Rashed · Moeenaldeen Al-Sayed

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Abstract Defects in the human gene encoding methylmalonyl-CoA mutase enzyme (MCM) give rise to a rare autosomal recessive inherited disorder of propionate metabolism termed mut methylmalonic acidemia (MMA). Patients with mut MMA have been divided into two subgroups: mut^0 with complete loss of MCM activity and mut^- with residual activity in the presence of adenosylcobalamin (AdoCbl). The disease typically presents in the first weeks or months of life and is clinically characterized by recurrent vomiting, metabolic acidosis, hyperammonemia, lethargy, poor feeding, failure to thrive and neurological deficit. To better elucidate the spectrum of mutations causing mut MMA in Saudi patients, we screened a cohort of 60 Saudi patients affected by either forms of the disease for mutations in the *MUT* gene. A total of 13 different mutations, including seven previously reported missense changes and six novel mutations, were detected in a homozygous state except for two compound heterozygous cases. The six novel mutations identified herein consist of three nonsense, two missense and one frameshift, distributed throughout the whole protein. This study describes for the first time the clinical and mutational spectrum of mut MMA in Saudi Arabian patients.

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F. Imtiaz · B.M. Al-Mubarak · A. Al-Mostafa · M. Al-Hamed · R. Allam
Department of Genetics, King Faisal Specialist Hospital and Research Centre, PO Box 3354, Riyadh 11211, Saudi Arabia

Z. Al-Hassnan · M. Al-Owain · H. Al-Zaidan · Z. Rahbeeni · A. Qari · M. Al-Sayed (✉)
Department of Medical Genetics, King Faisal Specialist Hospital and Research Centre, PO Box 3354, Riyadh 11211, Saudi Arabia
e-mail: moeen@kfshrc.edu.sa

E.A. Faqeih · A. Alasmari
Department of Pediatrics, King Fahad Medical City, Riyadh, Saudi Arabia

F. Al-Mutairi
Division of Genetics, Department of Pediatrics, Prince Sultan Medical Military City, Riyadh, Saudi Arabia

M. Alfadhel · W.M. Eyaid
Division of Genetics, Department of Pediatrics, King Saud bin Abdulaziz University for Health Sciences, Riyadh, Saudi Arabia

M. Alfadhel · W.M. Eyaid
Division of Genetics, Department of Pediatrics, King Saud bin Abdulaziz University for Health Sciences, Riyadh, Saudi Arabia

M.S. Rashed
Pharmagene Specialized Analytical Services, Aman Street, Cairo, Egypt

Introduction

Methylmalonic acidemia (MMA, OMIM 251000) is a common inborn error of organic acid metabolism occurring with a worldwide incidence rate ranging between 1:50,000 and 1:100,000 (Coulombe et al. 1981; Lemieux et al. 1988; Sniderman et al. 1999; Chace et al. 2001; Shigematsu et al. 2002; Sakamoto et al. 2007) and is inherited in an autosomal recessive manner (Matsui et al. 1983; Fenton 1995; Fenton et al. 2001). This disorder is caused by genetic defects in *MUT*, the gene encoding for L-methylmalonyl CoA mutase (MCM, EC 5.4.99.2), which catalyses the

conversion of L-methylmalonyl-CoA to succinyl-CoA utilizing adenosylcobalamin (AdoCbl) as a co-factor. Unconverted methylmalonyl-CoA is subsequently hydrolyzed to free coenzyme A (CoA) and methylmalonic acid (MMA), leading to accumulation of MMA in tissue and body fluids of affected individuals (Kovachy et al. 1983; Fenton et al. 2001). The exact incidence of MMA among live births in Saudi Arabia is not known; however, newborn screening results suggest that 1 in every 12,178 live newborns may be affected with this disease of which the majority are due to mutase deficiency (unpublished data). This is considered high when compared to the worldwide frequency, but it is not surprising due to the high rate of consanguineous marriages in Saudi Arabia.

Patients harbouring defects in the *MUT* gene have been distinguished by two biochemical criteria. Mutants with residual mutase activity in cell homogenates under saturating AdoCbl conditions, and whose ability to incorporate [1-14C] propionate is responsive to hydroxocobalamin supplementation of the culture medium, are designated as mut-, whereas those with no residual activity and no response of propionate incorporation to hydroxocobalamin are designated as mut^0 (Willard and Rosenberg 1980; Thoma and Leadlay 1996; Fowler et al. 2008). Mut^0 patients manifest as early as the neonatal period with poor feeding, vomiting, lethargy, hypotonia, altered level of consciousness, life-threatening metabolic ketoacidosis and moderate to severe hyperammonemia. If patients are not treated early and aggressively, the disease progresses to coma, neurological damage especially involving the basal ganglia and death in some cases (Lempp et al. 2007). Mut^- patients have a milder phenotype and present within the first 1–2 years of life (Martinez et al. 2005). Accumulation of MMA is associated with dysfunction of the mitochondrial respiratory chain reaction characterized by reduced ATP production and increased oxidative stress (Matsui et al. 1983; Fenton 1995). Evidence of respiratory chain impairment and/or oxidative stress was reported in MMA patients (Chandler et al. 2009; Ribas et al. 2012). Several complications have been described in long-term survival patients including neurodevelopmental delay, basal ganglia abnormalities, progressive renal failure, recurrent pancreatitis, recurrent bone marrow suppression and cardiomyopathy (Baumgarter and Viardot 1995; Nicolaides et al. 1998; Horster et al. 2007).

The human *MUT* gene was mapped to chromosome 6, consisting of 13 exons and spanning over 35 kb (Nham et al. 1990). The nuclear-encoded MUT is synthesized as a 750 amino acid long cytoplasmic precursor, bearing a 32 amino acid mitochondrial leader sequence cleavable upon transport into the mitochondria, where it homodimerizes with another cleaved precursor forming the mature enzyme. The human MUT primary structure has two major domains, as revealed by X-ray crystal structure and homology modelling studies, connected via a small interdomain linker: the N-terminal $(\beta/\alpha)_8$ barrel domain accommodating the substrate binding site and the C-terminal AdoCbl-binding domain, with the active site residing at the interface between these domains (Thoma and Leadlay 1996; Froese et al. 2010).

To date, 243 pathogenic mutations have been identified in the human *MUT* gene in various populations (HGMD®: http://www.hgmd.cf.ac.uk/ac/index.php). This study is the first to report a total of 14 mutations, 8 of which are novel in a cohort of 60 patients with mut MMA in Saudi Arabia. Three are believed to be founder mutations as all affected families originate from specific geographical locations in Syria and Saudi Arabia.

Materials and Methods

Patients and MMA Diagnosis

This study includes samples from 60 patients (from 56 different nuclear families) with mut MMA. Patients were ascertained through three sources: (1) index cases where the diagnosis was established based on the clinical presentation, abnormal acylcarnitine profile and urine organic acids, (2) siblings of index cases who were born and tested positive for MMA and (3) state-based newborn screening. Patients were recruited as part of an institutionally approved research project (RAC# 2020 011); informed consent was obtained, which adhered to the institutional guidelines and to the tenets of the Helsinki Declaration of 1975, as revised in 2000. Subsequently as diagnostic molecular testing was established for *MUT* gene locally, patients with clinical and biochemical diagnosis of MMA had routine genetic testing for mutation identification. Mutations in the *MUT* gene were identified in all patients.

Mutation Detection

Genomic DNA from all affected individuals was extracted from whole blood using the conventional salting-out method. Intronic primers were designed using the Primer3 program (http://frodo.wi.mit.edu/primer3/) to flank each of the 12 coding exons of *MUT* (primer sequences and conditions are available on request). PCR reactions for all patients and subsequently normal control samples were typically performed in a 25 μL reaction volume containing standard reagents and 10 ng of genomic DNA. Sequencing reactions were desalted and unincorporated nucleotides removed using ethanol precipitation and re-suspended in a formamide EDTA solution for injection on a MegaBACE 1000 DNA Analysis System (Molecular Dynamics; Sunnyvale, CA, USA). Purified PCR products covering the entire

coding region of *MUT* (accession no. ENSG00000146085) as identified on Ensembl (http://www.ensembl.org/index.html) were directly sequenced with the dideoxy chain-termination method using an ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) following the manufacturer's instructions. Sequence analysis was performed using the SeqMan 6.1 module of the Lasergene (DNA Star Inc. WI, USA) software package and then compared to the reference GenBank sequence (accession no. # NM_000255.3). Numbering commenced with the A of the ATG initiation codon as +1.

Results

The identified mutations and their associated phenotypes are summarized in Table 1. All patients except one came from consanguineous marriage. Where genomic DNA from parental and unaffected siblings was available, the molecular analysis was performed as described. All parents were heterozygous carriers and unaffected siblings were heterozygous carriers or wild-type normal. Briefly, the majority of index cases presented had an early neonatal presentation. Patients with Y110C mutation had a variable age of presentation ranging from neonatal to early childhood. Complications observed in childhood included growth delay, neurodevelopmental delay, chronic renal impairment and recurrent pancreatitis. Table 1 summarizes the identified mutations and their incidence.

In the current study we investigated the molecular background of mut MMA in a cohort of 60 patients, whereby the entire coding region and intron-exon boundaries of *MUT* were directly sequenced in both the forward and reverse direction using genomic DNA. In total, we have identified 13 different mutations in these 60 patients. Six of these mutations were novel, while the remaining seven have been previously described. Novel mutations included two missense mutations (c.329A>C resulting in p.Y110C and c.2075T>C resulting in p.L692P), three nonsense mutations (c.88C>T resulting in p.Q30*, c.109C>T resulting in p.Q37* and c.2200C>T resulting in p.Q734*) and one frameshift mutation (c.810_811delGGinsA p.A271LfsX11). None of the six novel mutations were reported in the locus-specific mutation databases such as the Human Gene Mutation Database Professional 2013 (http://www.hgmd.org) and the National Center for Biotechnology Information (NCBI) SNP database (http://www.ncbi.nlm.nih.gov/SNP), nor were they present in 300 chromosomes from ethnically matched normal controls suggesting that these variants are not population-based polymorphisms. Moreover, in silico analysis performed using a suite of bioinformatics tools including PolyPhen (http://genetics.bwh.harvard.edu/pph2/) and MutationTaster (http://www.mutationtaster.org/) revealed that all the novel missense mutations are predicted to probably be disease-causing further confirming pathogenicity. However, expression studies are required to validate this notion. Furthermore, alignment of orthologous protein sequences from human, monkey, mouse, dog and zebrafish obtained from Ensembl or UCSC Genome browsers (http://genome.ucsc.edu/cgi-bin/hgGateway) using the Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clustalo/) demonstrated a strong cross-species conservation of the novel missense mutations (p.Y110C and p.L692P) (Fig. 1). Seven different previously reported missense mutations were also identified in this study (p.R93H, p.R108C, p.F174S, p.G215S, p.Y364S, p.T387I and p.R694W). All the mutations were homozygous with an exception of two compound heterozygote cases harbouring the known p.R108C in combination with the novel p.Q37* mutation.

Among the 60 patients studied, p.R93H was the most prevalent mutation accounting for 35% of the cases. Interestingly, the next most common mutation was the novel p.Y110C missense mutation present in 25% of the cases. Q37* and Q734* mutations were equally observed.

Discussion

This study describes the spectrum of mutations in the *MUT* gene among 60 patients diagnosed with mut MMA in Saudi Arabia based on abnormal acylcarnitine profile and urine organic acids. It is important to note that there was a further patient in whom the *MUT* gene was sequenced and clear from mutation upon analysis. A deleterious mutation was subsequently identified in the *MMAA* gene (NM_172250) for this single case. Patients with methylmalonic aciduria types MMAB and MMADHC have not been found. All of the mutations reported here occurred in a homozygous state with the exception of two cases being compound heterozygous for p.R108C in association with the novel p.Q37* mutation, reflecting the consanguineous nature of the Saudi population. Considering the wide spectrum of mutations (nonsense, missense and frameshift), their distribution is heterogeneous with the majority clustering in the substrate-binding $(\beta/\alpha)_8$ barrel and the AdoCbl-binding domains. Six different previously reported missense mutations (p.R93H, p.R108C, p.F174S, p.G215S, p.Y364S and p.R694W) have been detected in our cohort of patients. Four of which (p.R93H, p.R108C, p.F174S and p.G215S) were located within the substrate-binding $(\beta/\alpha)_8$ barrel domain and one (p.R694W) residing in the AdoCbl-binding domain. The R93H mutation, detected in 35% of the cases, was first identified in a homozygous state in a cell line derived from a Caucasian patient with mut^0 MMA (Raff et al. 1991). Studies have revealed that p.R93H-expressing cells when

Table 1 Identified *MUT* mutations and phenotypes of the *mut* MMA patients

Mutation	Genotype	Amino acid change	Domain	Number of patients with the genotype	Age of onset	Renal impairment	Growth delay	Pancreatitis	References
c.88C>T	Homozygous	**p.Q30***	ML	1	Neonatal	Not observed[a]	Yes	Not observed	This study
c.109C>T	Homozygous	**p.Q37***	NT	4	Neonatal	Present	Yes	Observed	This study
c.278 G>A	Homozygous	p.R93H	$(\beta/\alpha)_8$	21	Neonatal	Present	Yes	Observed	Raff et al. (1991)
c.322C>T	Homozygous	p.R108C	$(\beta/\alpha)_8$	1	Neonatal[b]	Unknown	Unknown	Unknown	Worgan et al. (2006)
c.329 A>G	Homozygous	**p.Y110C**	$(\beta/\alpha)_8$	15	Neonatal (12)/ infantile (3)	Present	Variable	Observed	This study
c.521 T>C	Homozygous	p.F174S	$(\beta/\alpha)_8$	1	Neonatal	Not observed[a]	Yes	Not observed[a]	Fuchshuber et al. (2000)
c.643 G>A	Homozygous	p.G215S	$(\beta/\alpha)_8$	1	Neonatal	Not observed[a]	Yes	Not observed[a]	Acquaviva et al. (2005)
c.810_811delGGinsA	Homozygous	**p.A271LfsX11**	$(\beta/\alpha)_8$	2	Neonatal	Present	Yes	Observed	This study
c.1091 A>C	Homozygous	p.Y364S	$(\beta/\alpha)_8$	1	Neonatal	Present	Yes	Observed	Gradinger et al. (2007)
c.1160C>T	Homozygous	p.T387I	$(\beta/\alpha)_8$	1	Neonatal	Not observed[c]	Yes	Not observed[c]	Dundar et al. (2012)
c.2075 T>C	Homozygous	**p.L692P**	AdoCbl	1	Neonatal	Yes	Yes	Not observed[a]	This study
c.2080C>T	Homozygous	p.R694W	AdoCbl	5	Neonatal	Not observed[a]	Yes	Observed	Lempp et al. (2007)
c.2200C>T	Homozygous	**p.Q734***	AdoCbl	4	Neonatal	Present	Yes	Observed	This study
c.88C>T/c.322C>T	Compound heterozygous	**p.Q37***/p.R108C	NT/$(\beta/\alpha)_8$	2	Neonatal	Present	Yes	Observed	This study/ Worgan et al. (2006)

Novel mutations are in bold

ML mitochondrial leader sequence, *NT* N-terminal extended segment, $(\beta/\alpha)_8$ substrate-binding $(\beta/\alpha)_8$ barrel, *AdoCbl* AdoCbl-binding domain

[a] Patients are still young (complications may develop later)

[b] Unable to obtain data as patient not followed at our institute

[c] Long follow-up over 19 years

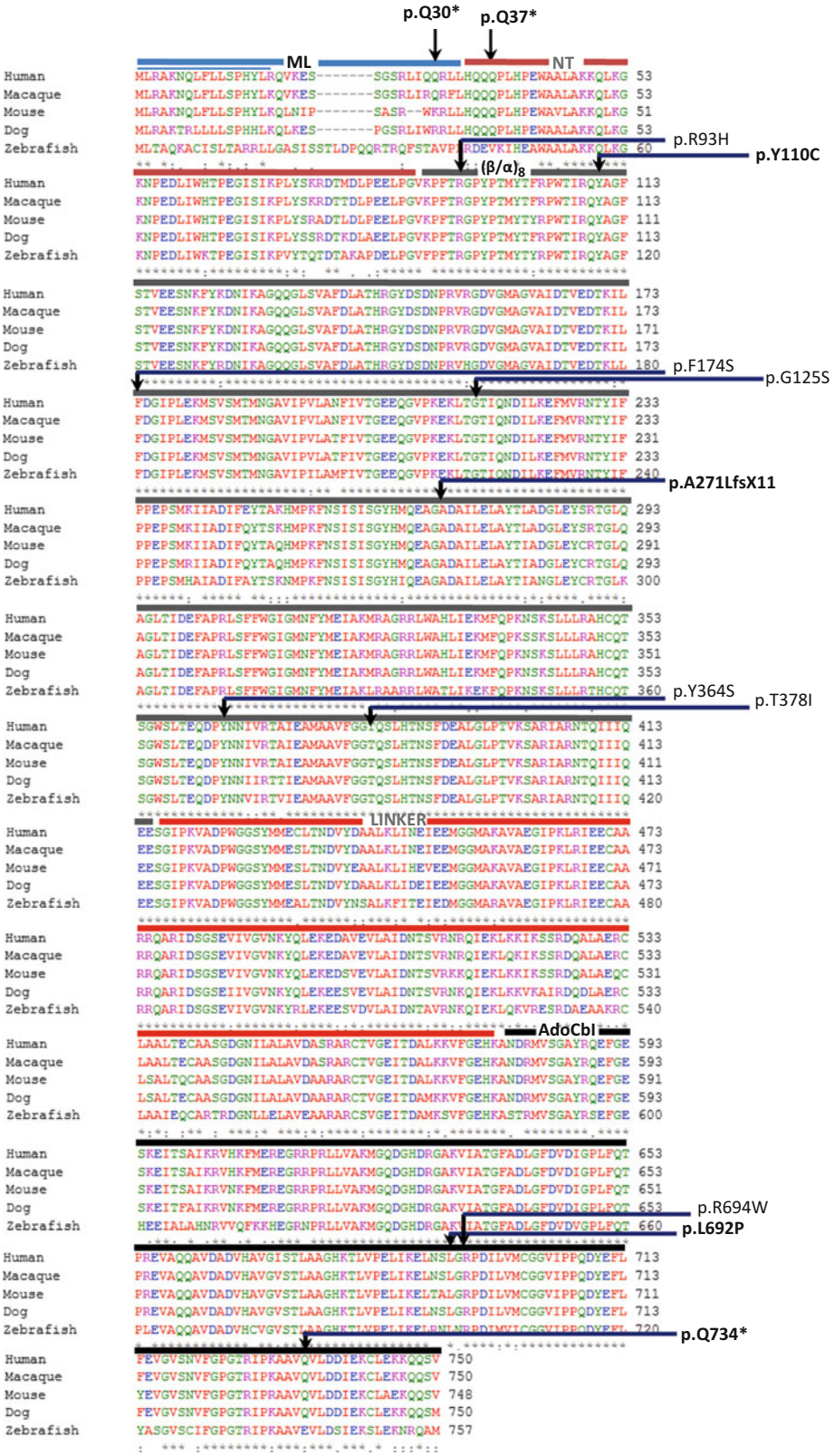

Fig. 1 Cross-species conservation of MUT protein between human (NP_000246.2), macaque (XP_005552835.1), mouse (AAH19175.1), dog (XP_532164.3) and zebrafish (AAI39861.1). ML, mitochondrial leader sequence; NT, N-terminal extended segment; $(\beta/\alpha)_8$, substrate-binding $(\beta/\alpha)_8$ barrel; Linker, interdomain linker region; AdoCbl, AdoCbl-binding domain. Novel mutations are in bold, (*) denotes amino acids identical in all sequences, (:) denotes conserved substitutions and (.) denotes semi-conserved substitutions. Alignment performed by ClustalOmega (http://www.ebi.ac.uk/Tools/msa/clustalo/) using protein sequences from the National Centre of Biotechnology (NCBI)

co-transfected with clones bearing one of the other MUT mutations such as p.R694W, p.G648D and p.G626C or fused with cells expressing either of these mutations have the capacity to produce significant levels of enzyme activity as a result of interallelic complementation (Crane and Ledley 1994; Qureshi et al. 1994). Among Japanese patients, the p.R93H mutation was recurrent in compound heterozygous patients associated with other mutations (Kobayashi et al. 2006). Unlike Japanese patients, all Saudi patients harbouring the p.R93H in this study were homozygous for the mutation. It is likely that this is a founder mutation as all affected patients came from unrelated families from the southern region of Saudi Arabia. The mutation was observed to be associated with a severe early-onset phenotype. All patients uniformly had growth retardation, progressive renal disease, cognitive delay and recurrent pancreatitis. The next most common mutation was the novel p.Y110C missense mutation present in 25% of the cases. Again this mutation is expected to be a founder as all families affected with mutation had the same tribal orientation. Interestingly, this mutation has a more variable phenotype with age of onset ranging from neonatal to early childhood. Growth delay was also variable with some patients having normal growth and others with significant growth delay. In addition, cognitive function has also ranged from normal to moderate cognitive delay. Next were p.Q37* and p.Q734* mutations and both were equally observed. The first one affected unrelated families with roots back to a specific region in Syria near Damascus, and the second was identified in two siblings from a specific tribe in addition to two more patients. As predicted these two mutations result in a severe early-onset disease as p.Q37* introduces a termination codon at the start of the NT extended segment. The consequence of such mutations was first described by Ledley et al., whereby a nonsense mutation at position 17 terminated translation from the original AUG and reinitiated translation at an in-frame AUG codon internal to the mature protein sequence producing immunoreactive truncated protein (Ledley et al. 1990). The truncated protein lacking leader peptide and a portion of the amino terminus of the mature apoenzyme remains in the cytoplasm and undergoes degradation (Fenton et al. 1987). Recently, more termination mutations have been identified: One (p.Q7*) was found in a European patient occurring early in the sequence (Acquaviva et al. 2005) and the other (p.Q31*), adjacent to the one reported in our study (p.Q30*), was detected in a Thai patient (Vatanavicharn et al. 2012). Both mutations were predicted to result in the absence of functional gene product. p.Q734* was found in the AdoCbl-binding domain causing the loss of 16 amino acid residues in the C-terminus. Although the substrate-binding domain and most of the AdoCbl-binding domain are intact, patients with the p.Q734* mutation exhibited a phenotype reminiscent of many mut^0 patients suggesting that the last 16 amino acid residues are indispensable for the enzyme function. This observation is in agreement with previous reports on patients carrying another stop codon (p.Q727*), located 7 amino acids upstream of p.Q734*, which have been diagnosed with mut^0 form of MMA (Kobayashi et al. 2006; Worgan et al. 2006; Dundar et al. 2012) supporting the notion that such mutations can be detrimental to enzyme function.

The third most recurrent known mutation in our population is p.R694W, identified in four patients. Patients affected with this genotype range from 2 to 11 years of age. So far they do not show any signs of renal involvement and have growth delay, and pancreatitis was observed once only in one patient. Therefore, this genotype might be associated with a milder phenotype. This is consistent with previous reports of this mutation (Janata et al. 1997; Acquaviva et al. 2005). The rest of the mutations were seen in single cases and the associated clinical findings are summarized in Table 1. As predicted, p.Q30* which introduces an early termination codon at position 30 (p.Q30*) within the mitochondrial leader sequence is associated with a severe phenotype.

One of the two novel missense mutations identified in the present study (p.Y110C) was mapped to the substrate-binding $(\beta/\alpha)_8$ barrel domain and the other (p.L692P) was mapped to AdoCbl-binding domain, both affecting highly conserved amino acids. The preservation of these amino acids along with the absence of these missense changes in normal controls makes it very likely that these mutations are pathologically significant.

The p.Y110C mutation lies within the 1st β-strand of the substrate-binding domain. This domain is thought to be responsible for the binding of the CoA ester substrate (Thoma and Leadlay 1996); therefore, the substitution of the aromatic amino acid (tyrosine 110) which points directly to the active site with an uncharged polar residue (cysteine) may substantially affect activity. The second missense mutation resulted in a non-conservative amino acid substitution (p.L692P) within the AdoCbl-binding domain would most likely suffer from a breaking of the secondary structure (alpha-helix) of which the leucine is part of.

Finally, one frameshift mutation (p. Ala271LeufsX11) was found in the substrate-binding $(\beta/\alpha)_8$ barrel domain resulting in a premature stop codon. Predictably, such mutations would abolish the enzyme activity via mechanisms involving nonsense-mediated mRNA decay or elimination of truncated proteins.

In conclusion, we have detected a total of 13 different mutations, six of which were novel mutations, including three nonsense mutations, two missense mutations and one frameshift mutation, in 60 Saudi patients, in addition to seven previously reported mutations. The diversity of *MUT*

gene mutations detected in our patients suggests the pleomorphic nature of this condition in the Saudi population. Homoallelic mutations are almost universally observed in our cohort, due to the extensively consanguineous nature of the Saudi population, negating clinical heterogeneity resulting from interallelic complementation. Our study summarizes the spectrum of mutations in the *MUT* gene in Saudi Arabia. It provides useful genotype phenotype correlation that will help in predicting clinical outcome and genetic counselling of families affected with this disease. Using the presented information, rapid molecular diagnosis can be established and preventative reproductive counselling such as prenatal diagnosis, preimplantation genetic diagnosis and carrier testing can be implemented.

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Compliance with Ethics Guidelines

Informed Consent: All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients being included in the study.

FI, BAM, AM, MH and RA performed molecular genetic studies, analysis and interpretation. ZH, MO, HZ, ZR, AQ, EF, AA, FM, MF, WE, MS and MAS provided patient information, clinical diagnosis and samples and were involved in data interpretation. All authors were all involved in drafting and revising the article.

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