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Research paper

Molecular, biochemical, and structural analysis of a novel mutation in patients with methylmalonyl-CoA mutase deficiency

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ABSTRACT

Background: Methylmalonic aciduria (MMA) is an inborn error of metabolism resulting from genetic defects in methylmalonyl-CoA mutase (MCM). This enzyme is encoded by the MUT gene and is required for the degradation of odd-chain fatty acids, the amino acids valine, isoleucine, methionine, and threonine, and cholesterol. *Method:* Three unrelated affected patients with isolated MMA and their parents were studied. The MUT gene was analyzed by PCR and sequencing of its entire coding region and the highly conserved exon-intron splice junctions. The homology modeling of the novel mutation found in the MUT gene was performed using the online Swiss-Prot server for automated modeling and then analyzed with special bioinformatics software to better study the structural effects caused by the mutation.

Result: We found one homozygous nucleotide change in intron 12 of the *MUT* gene (c.2125-3 C > G). The variant is located near the highly conserved acceptor splice site of intron 12. A region at the C-terminus of the protein from ASP709 to GLN748 has been deleted by the alteration of c.2125-3 C > G in intron 12 of the MUT gene. Further studies of the novel mutation in the *MUT* gene by means of homology modeling revealed abnormalities in the protein's structure, which causes the protein to act malfunctioning and also the mRNA expression analysis of *MUT* gene confirmed these results.

Conclusion: We report this novel mutation, including its clinical and biochemical features and genetic defects, in the *MUT* gene of three patients affected with isolated MMA. Structural analyses of the mutated protein identified changes in the energy and stereochemical features of the protein that unfortunately altered the protein's functionalities. Therefore, we demonstrate that a novel splice site mutation in intron 12 of the *MUT* gene is a potential highly pathogenic allele via inhibition of alternative splicing.

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1. Introduction

Methylmalonic aciduria (MMA) is an autosomal recessive inherited inborn error of metabolism due to the impaired isomerization of L-methylmalonyl-CoA to succinyl CoA during the oxidation of propionate towards the Krebs cycle (Fowler et al., 2008). This reaction is catalyzed by a cobalamin-dependent mitochondrial enzyme named L-methylmalonyl-CoA mutase (MCM, EC 5.4.99.2) using adenosylcobalamin (Adocbl) as a cofactor (Retey, 1982;

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http://dx.doi.org/10.1016/j.gene.2015.10.002 0378-1119/© 2015 Elsevier B.V. All rights reserved. Martínez et al., 2005; Peters et al., 2002). The functional defect of L-methylmalonyl-CoA mutase is caused by the enzyme deficiency (mut gene) or by a defect in metabolism (cbl A) or transport (cbl B) of its cofactor, deoxyadenosyl cobalamin (Rosenblatt and Fenton, 2001; Dobson et al., 2002a, 2002b). Two classes of mutation in MCM are identified, mut⁰ with no detectable MCM activity and mut⁻ with partial MCM activity (Ledley et al., 1988; Rosenberg and Fenton, 1989). The incidence of MMA estimated by a survey of newborns in Massachusetts is 1 in 48,000 births (Coulombe et al. 1981). Because of high rate of consanguineous marriage in Iran, the prevalence of this disorder might be high.

The human *MUT* gene maps to chromosome region 6p12-21.2 (Ledley et al., 1988) and has 13 exons spanning over 35 kb of genomic DNA (Nham et al., 1990; Chandler and Venditti, 2005; Lempp et al., 2007). The gene is transcribed as a 2.7 kb mRNA. The expressed mRNA encodes a 750 amino acid including a 32 amino acid mitochondrial targeting sequence which is cleaved during transport into the

Abbreviations: bp, Base pair; cDNA, DNA complementary to RNA; dNTP, deoxyribonucleoside triphosphate; MMA, Methylmalonic Aciduria; MCM, Methylmalonyl CoA mutase; HGMD, Human gene mutation database; mRNA, Messenger RNA; GC/MS, Gas chromatography/mass spectrometry; PCR, Polymerase chain reaction; RT-PCR, Reverse transcriptase; PA, Propionic aciduria.

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F. Keyfi et al. / Gene xxx (2015) xxx-xxx

mitochondrion and a mature enzyme is formed. The mature enzyme is a homodimer with the N-terminal CoA binding domain and the C-terminal cobalamin-binding domain (Nham et al., 1990; Fenton et al., 2001). Currently, various studies have identified more than 250 different disease-causing mutations in the *MUT* gene.

This enzyme is required for the degradation of odd-chain fatty acids, the amino acids valine, isoleucine, methionine, and threonine, and cholesterol (Martínez et al., 2005).

L-methylmalonyl-CoA mutase deficiency is characterized by recurrent episodes of vomiting, lethargy, profound ketoacidosis, hyperammonemia, and pancytopenia in infancy, resulting in failure to thrive, mental retardation, and early death. Between decompensation episodes plasma carnitine remains low, glycine is elevated, and there is massive excretion of methylmalonic acid in urine. Complications include cardiomyopathy, metabolic stroke, pancreatitis, and progressive renal failure (Dündar et al., 2012).

In the present study we report the clinical and biochemical features and genetic defects in the *MUT* gene of three patients affected with isolated MMA. As a part of the in-silico study, the deduced novel mutation has been modeled using online Swiss-Prot server for automated modeling and improved by recent bioinformatics software and techniques. Structural abnormalities of the resulting amino acid deletions, as predicted from molecular modeling, are compatible with the observed phenotype. We demonstrate that a novel splice site mutation in intron 12 of the *MUT* gene is a potential highly pathogenic allele via inhibition of alternative splicing.

2. Patients and methods

2.1. Patients and MMA diagnosis

Three unrelated affected patients with isolated MMA and their parents were studied by the Genetic Counseling Services in Pardis Clinical and Genetics Laboratory. All procedures followed were in accordance with the ethical standards of the committee on human experimentation MUMS (Mashhad University of Medical Science) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study. Upon reviewing, the Ethical Committee approved the above-mentioned protocol in its session hold on October 14, 2012. The methodological aspect of the research was in agreement with ethical principles. All patients were diagnosed by urine organic acid analysis using urease treatment extraction with gas chromatography–mass spectrometry (GC–MS) after presenting with clinical symptoms and high levels of propionylcarnitine (µmol/L) through-tandem mass spectrometry. All patients had increased urinary excretions of methylmalonate with or without 3-hydroxypropionate and methylcitrate.

2.2. Mutation analysis

Genomic DNA was extracted from peripheral blood samples by the salting out method (Miller et al., 1988). Samples with purities of 1.5–1.8 (260 nm/280 nm ratio) and final concentrations of 5 ng/ μ L were used for amplifications.

The MUT gene was analyzed by PCR and sequencing of both DNA strands of the entire coding region and the highly conserved exonintron splice junctions. The reference sequence of the *MUT* gene is NM_000255.3. To analyze this novel mutation, oligonucleotide primers that hybridized to flanking intronic sequences of exon 13 of the MUT gene (Forward: 5'-ACC ACA AGC AAC ACA TAG AG-3', Reverse: 5'-TCA ATA TCA TCA AGC ACC TGA A-3') were designed. Primers were designed using Becon Designer, V 7.51 Primer Analysis Software. The reaction mixture to amplify the entire exons consisted of 50 ng of genomic DNA, 250 nmol/L each of the sense and antisense primers, 200 µmol/L each of dATP, dCTP, dGTP, and dTTP, and 1 unit of Taq polymerase in a total volume of 20 µl in the manufacturer's reaction buffer. The PCR conditions consisted of an initial denaturation at 95 °C for 5 min, followed by 40 cycles at 95 °C for 30 s, 56 °C for 30 s, and a 30 s extension at 72 °C. A final extension was performed at 72 °C for 5 min. Amplification was confirmed by electrophoresis of 2 µL of amplified product on a 1.5% agarose gel in $1 \times TAE$ stained with ethidium bromide. At least two PCR products were directly sequenced using the oligonucleotide primers that had been employed for PCR amplification. Products of the



Fig. 1. Family pedigree and mutation spectrum of three patients with MMA (A: Patient 1, B: Patient 2, C: Patient 3).

F. Keyfi et al. / Gene xxx (2015) xxx-xxx

sequencing reaction were analyzed using DNA Sequencher 4.7. The phase of the novel mutation was confirmed by sequencing DNA samples from the patients' parents. This mutation has been recorded in GenBank database with accession no: KF030882.

2.3. Structural analysis

To model the newly-identified mutated protein, the mutated amino acid sequence was first compared to the similarity databases of templates for modeling using the online server Swiss-Prot Template Identification tool. Afterwards the template most similar to the target mutated protein with 99.7% sequence identity (Accession code: 2xiq) was chosen for the modeling (Zdobnov and Apweiler, 2001).That template has been solved to 1.95 Å.

The 2xiq template was submitted for the homology modeling job using the online Swiss-Prot server (Arnold et al., 2006; Guex and Peitsch, 1997 and Schwede et al., 2003) and using ZMM software, the result has been set for the Energy Minimization. The ZMM software uses the Amber all-atom force field to minimize the conformational energy including torsion and bond angles with a cut-off distance of 10Å (Weiner et al., 1984). Low-energy conformation was reached by the Monte Carlo Minimization Method (Li and Scheraga, 1987). To improve the lowest-energy conformation, the Energy Minimization was terminated after 100 sequential minimizations.

The essential accuracy and correctness of the model was evaluated using the PROCHECK (Laskowski et al., 1993) and WHAT-IF (Vriend, 1990) web servers from the online server http://nihserver.mbi.ucla. edu/SAVES/, and the results confirmed the abnormalities caused by the structural deficiencies which have been occurred due to cutting region of (ASP709–GLN748) including the C-terminus.

2.4. mRNA expression analysis

The effect of the splicing mutation was analyzed by RT-PCR. Total cellular RNA was purified from leukocytes extracted from whole blood using TRIZOL (Invitrogen). First-strand cDNA was generated from total RNA using oligo-dT, followed by PCR amplification with RNA-specific primers for both N-terminal (exons 5–6, primer F: 5'-GGGAATTGGAAT GAATTTCTATA-3', Reverse: 5'- TAAAGCAGCATCATAAACATC-3) and C-terminal (exon 12 to 3'UTR, location of splice site mutation, primer Forward: 5'-CTCATCAAAGAACTTAACTCCCT-3', Reverse: 5'- CTTATAGC ATGACACCAGGC-3') designed using Becon Designer, V 7.51 Primer Analysis Software. Fragments were electrophoresed on agarose gels and were visualized by ethidium bromide staining.

3. Result and discussion

3.1. Clinical features

Three unrelated affected patients with isolated MMA and their parents were studied. The pedigrees of the three families are shown in Fig. 1. The clinical features of the patients are presented below:

Patient 1, a female infant who was a product of a consanguineous marriage, presented at 22 months of age with congenital malformations, seizures, hypotonia, high acidosis, vomiting, and constipation. The clinical outcome was developmental delay, abnormality in walking, and inability to speak. Tandem mass spectrometry analysis showed significant elevation of propionylcarnitine (9.8 µmol/L), which may be indicative of MMA or propionic acidemia (PA). But urine organic acid analysis indicated increased excretion of methylmalonic and methylcitric acids. The patient was treated for MMA with phenobarbital, L-carnitine and vitamin B12, and died at the age of 3 years.

Patient 2, a female infant who was a product of a consanguineous marriage, presented at 18 months of age with weakness, lethargy, milk intolerance, vomiting, anorexia, and swallowing and breathing problems. The clinical outcome was failure to thrive, developmental

Carnitine, 2.5 mL/day vitamin B12, 1.0 mg/2 mg/day L-carnitine, 5 mL/day vitamin B12, mg/day injection biotin, 3 mg/day sodium Carnitine, 10 mL/day vitamin B12, 1.0 lay injection biotin, 3 mg/day only in lethargy situation) penzoate, 250 mg/day 1.0 mg/day injection ^{phenobarbital}, Treatment Urine MMA (AT) (µmol/mmol creatinine) 47.0 40.0 35.0 Urine MMA (BT) (µmol/mmol creatinine) Urine MMA: urine methylmalonic acid, parents Consan.: parents consanguinity, PC: propionyl carnitine (C3), BT: before treatment, and AT: after treatment. 2800 2200 1950 <u>ອ</u> µmol/L 4.24 9.8 61.0 PC Term Term Term Age at birth 2.650 kg 2.450 kg 2.90 kg weight Birth Current 6 years Died Died age Siblings brother brother ů infection, severe breathing, Died at the age of 3.5 years Died at the age of 3 years infection, seizures) cardiac arrest) Outcome Alive children died Family history Clinical characterization of isolated MMA patients. Yes-1 ۶ ۶ Parents consan. Yes Yes Yes Age of diagnosis 22 months months Female 3 days 18 Female Female Sex Patient \sim ŝ

Table 1



Fig. 2. A sequencing chromatogram of family patient 3 (A) sequenogram of patient 3 with the homozygous c.2125-3C > G mutation, (B) sequenogram of father's patient 3 with the heterozygous c.2125-3C > G mutation, (C) sequenogram of mother's patient 3 with the heterozygous c.2125-3C > G mutation, (C) sequenogram of mother's patient 3 with the heterozygous c.2125-3C > G mutation, (C) sequenogram of mother's patient 3 with the heterozygous c.2125-3C > G mutation, (C) sequenogram of mother's patient 3 with the heterozygous c.2125-3C > G mutation, (C) sequenogram of mother's patient 3 with the heterozygous c.2125-3C > G mutation, (C) sequenogram of mother's patient 3 with the heterozygous c.2125-3C > G mutation, (C) sequenogram of mother's patient 3 with the heterozygous c.2125-3C > G mutation, (C) sequenogram of mother's patient 3 with the heterozygous c.2125-3C > G mutation, (C) sequenogram of mother's patient 3 with the heterozygous c.2125-3C > G mutation, (C) sequenogram of mother's patient 3 with the heterozygous c.2125-3C > G mutation, (C) sequenogram of mother's patient 3 with the heterozygous c.2125-3C > G mutation, (C) sequenogram of mother's patient 3 with the heterozygous c.2125-3C > G mutation, (C) sequenogram of mother's patient 3 with the heterozygous c.2125-3C > G mutation, (C) sequenogram of mother's patient 3 with the heterozygous c.2125-3C > G mutation, (C) sequenogram of mother's patient 3 with the heterozygous c.2125-3C > G mutation, (C) sequenogram of mother's patient 3 with the heterozygous c.2125-3C > G mutation, (C) sequenogram of mother's patient 3 with the heterozygous c.2125-3C > G mutation, (C) sequenogram of mother's patient 3 with the heterozygous c.2125-3C > G mutation, (C) sequenogram of mother's patient 3 with the heterozygous c.2125-3C > G mutation, (C) sequenogram of mother's patient 3 with the heterozygous c.2125-3C > G mutation, (C) sequenogram of mother's patient 3 with the heterozygous c.2125-3C > G mutation, (C) sequenogram of mother's patient 3 with the heterozygous c.2125-3C

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10

F. Keyfi et al. / Gene xxx (2015) xxx-xxx

delay, sleeping difficulties, encephalopathy, and abnormalities in sitting and speech. Tandem mass spectrometry analysis showed significant elevation of propionylcarnitine ($4.24 \mu mol/L$), which may be indicative of MMA or PA. But urine organic acid analysis indicated increased excretion of methylmalonic and methylcitric acids. The patient was treated for MMA with L-carnitine, biotin and vitamin B12, and died at the age of 3.5 years.

Patient 3, a female infant who was a product of a consanguineous marriage, presented at 3 days of age with rapid breathing, lethargy, milk intolerance, vomiting, anorexia, hypotonia, and high acidosis. The clinical outcome was failure to thrive, developmental delay, including delays in sitting and walking, encephalopathy, and constipation. Tandem mass spectrometry analysis showed significant elevation of propionylcarnitine (61 µmol/L), which may be indicative of MMA or PA. But urine organic acid analysis indicated increased excretion of methylmalonic and methylcitric acids. The patient was treated for MMA with L-carnitine, biotin, sodium benzoate and vitamin B12. She is now 6 years old with moderately delayed development. In addition to the proband, this family had an infant with similar symptoms as proband, however she died prior to molecular diagnosis.

The clinical features of the three patients are summarized in Table 1.

3.2. Mutation analysis

DNA samples of the three patients were analyzed for mutations in the *MUT* gene. We confirmed the mutation's homozygosity through analyses of the parental samples. We found one heterozygous variant in intron 12 of the *MUT* gene (c.2125-3C > G). This is identical to the mutation that was previously identified as homozygous in the affected children. A sequencing chromatogram of family patient 3 is shown in Fig. 2. The mutation is located at nucleotide 31,470 of the reference sequence of the *MUT* gene; NM_000255.3. This change, located near the exon/intron boundary, creates an AG dinucleotide, thus it may activate a cryptic splice site. Based on our results from affected patients, their parents, and unaffected siblings, we conclude that the three patients are likely carriers of a disease-causing mutation in the *MUT* gene. These splice site mutations could cause significant change in protein function and enzyme's activity.

3.3. Structural analysis

For the purpose of further investigating the accuracy of the model, it was analyzed in terms of stereochemical and geometrical parameters such as G-Factor, bond lengths, and bond angles. In addition, most of the residues were inside the desirable regions of the Ramachandran map.

3.4. mRNA expression analysis

Expression of MCM in leukocyte extracts from the patients and normal controls for N-terminal (exons 5–6) and C-terminal (exon 13, location of splice site mutation) was analyzed by RT-PCR and agarose gel electrophoresis. MCM expression in exons 5–6 was observed in samples from control and patients with the homozygous variant in intron 12 of the MUT gene (c.2125-3C > G, Fig. 3C, lanes 2 and 3), but expression of MCM in exon 13 was undetectable in samples from patients with the homozygous variant in intron 12 of the *MUT* gene (c.2125-3C > G, Fig. 3B, lane 2), whereas MCM expression was observed in samples from controls (Fig. 3B, lane 3). Therefore expressed protein is a truncated protein lacking exon 13 which code for 42 amino acids in protein C-terminal.

3.5. Discussion

To date, various studies have identified more than 250 different disease-causing mutations in the *MUT* gene, which include 172 missense or nonsense mutations, 24 splicing mutations, 34 small deletions, 16 small insertions, 3 small indels, and 1 gross deletion. All the mutations are available in the Human Gene Mutation Database (HGMD).

We found one homozygous nucleotide change in intron 12 of the MUT gene (c.2125–3 C > G). This variant has not been previously described in the literature. The variant is located near the highly conserved acceptor splice site of intron 12, which may influence splicing. By analysis of this mutation in the patients' parents and unaffected siblings, it should be possible to determine genotype-phenotype correlations and the clinical significance of the potential mutation. This mutation was found in the homozygous state in a total of three Iranian patients, all with a mut⁰ phenotype, suggesting a direct correlation between this particular genotype and a mut⁰ phenotype. These three patients had an early-onset disease, which showed initial symptoms at the first 24 months of life. The symptoms included lethargy, milk intolerance, vomiting, anorexia, hypotonia, and high acidosis. This mutation in homozygous form (c.2125–3 C > G) was mut⁰ with no detectable MCM activity. The lack of MCM activity in mut⁰ patients is probably due to the absence of 42 amino acids in cofactor binding domain. Structural analyses of the mutated protein also revealed major changes in its tertiary structure due to the deleted region (ASP709-



Fig. 3. Expression analysis of β-actin and methylmalonyl-CoA mutase. RNA's were isolated and cDNA was amplified by RT-PCR. Amplification products were electrophoresed on agarose gels and visualized with EtBr staining. (A) β-Actin (size: 113 bp): lanes, 1: 100 bp marker, 2: RNA from a patient with the c.2125-3C > G mutation, 3: RNA from a normal control, and 4: negative control. (B) Analysis of MCM (mut gene, exons 5–6, size: 403 bp): lanes, 1: 10 bp marker, 2: RNA from a patient with the c.2125-3C > G mutation, 3: RNA from a normal control, 4: negative control. (C) Analysis of MCM (mut gene, exons 5–6, size: 403 bp): lanes, 1: 1 kbp marker, 2: RNA from a patient with the c.2125-3C > G mutation, 3: RNA from a normal control, and 4: negative control.

6

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F. Keyfi et al. / Gene xxx (2015) xxx-xxx

GLN748) and cutting C-terminus that will affect Cbl-binding site which causes the protein dysfunction. It seems that the big alteration in the structure of the protein causes great impacts on the function of it due to the strong correlation between the protein tertiary structure and its function (Ogasawara et al., 1994).

In a similar study by Worgan LC et al. a novel mutation was reported in c.2125–1 G > A. This variant was identified in the Italian population with mut^0 class. This finding is consistent with our study and confirmed that these splice site mutations could cause significant change in protein function and enzyme's activity.

There is no history of molecular studies on this particular disease in the Iranian population. Karimzadeh P et al. described clinical symptoms and neuroimaging findings of 20 cases with this disorder. They showed that the patients with early diagnosis had a more favorable clinical response in growth index, refractory seizure, anorexia, and neurodevelopmental delay (Karimzadeh et al., 2013).

Based on these results, early detection of MMA is critical. Early diagnoses can prevent metabolic crises and improve patient survival (Shevell et al., 1993). A case in point, patient No. 3 who is diagnosed early (3 Days of birth), still lives, while the others have expired. Identification of mutations in MMA patients will facilitate differential confirmatory diagnosis, which is important to the implementation of appropriate treatments. It will also aid carrier detection, genetic counseling, prenatal diagnosis, and determination of the carrier status of the parents and their relatives, which could be helpful for their family planning.

4. Conclusion

We report this novel mutation, including its clinical and biochemical features and genetic defects, in the *MUT* gene of three patients affected with isolated MMA. Structural analyses of the altered protein identified changes in the energy level and stereochemical features of it that unfortunately altered the protein's functionalities. Therefore, we demonstrate that a novel splice site mutation in intron 12 of the *MUT* gene is a potential highly pathogenic allele via inhibition of alternative splicing.

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All of the authors declare that they have no conflict of interest.

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