3-methylglutaconic aciduria research

A distinct type of 3-methylglutaconic aciduria due to a mutation in the Translocase of Inner Mitochondrial Membrane 50 (*TIMM50*) gene

Abstract

BACKGROUND: 3-methylglutaconic aciduria biochemically characterized by increased urinary excretion of 3-methylglutaconic acid result from defective leucine metabolism and disorders affecting mitochondrial function though in many cases the cause remains unknown. Recently mutations in mitochondrial TIMM50 gene has been reported in four patients from two unrelated families. We report additional mutations in TIMM50 gene in 6 individuals from two unrelated consanguineous families with a distinctive type of 3-methylglutaconic aciduria.

METHODS: We report on three patients of South Asian ancestry with intractable epilepsy, microcephaly, developmental delay, visual deficit spastic quadriplegia and three Caucasian patients of eastern European origin with intellectual disability with or without seizure. Metabolic testing revealed mild lactic acidosis and excretion of large amount of 3-methylglutaconic acid in urine in all patients. Full exome sequencing was performed using genomic DNA isolated from one surviving patient, two healthy siblings and both parents of South Asian family. Exome sequencing was also performed for Caucasian patients of eastern European origin.

RESULTS: Exome sequencing identified two homozygous mutation Gly372Ser and Iso392Thr mutations in the gene TIMM50. There were no other candidate alterations in exome that could explain the phenotype in the proband. The mutations are located in the conserved C-terminal domain of the Tim50 protein that interacts with the N-terminal domain of the Tim23 protein in the

intermembrane space and regulates mitochondrial protein import of presequence-containing polypeptides Both parents are heterozygous.

CONCLUSION: Given the phenotypic similarilty of the patients from two unrelated families and an earlier report of mutations in additional family, we conclude that TIMM50 gene mutation results in a novel mitochondrial disorder with 3-methyl glutaconic aciduria.

INTRODUCTION

3-methylglutaconic aciduria (MGCA), an increase in urinary 3-methylglutaconic acid or 3-methylglutaric acid, can be a nonspecific finding in mitochondrial disorders, organic aciduria, urea cycles disease, neuromuscular disorders. but is a consistent abnormality of 3-methylglutaconyl-CoA hydratase deficiency and patients with mutations in *TAZ, SERAC1, OPA3, DNAJC19 and TMEM70* gene [1] . These genes all encode mitochondrial membrane or membrane related proteins. In 3-methylglutaconyl-CoA hydratase deficiency due to mutation in *AUH* gene , 3-methylglutaconic acid derives from 3-methylglutaconyl CoA (3MG CoA), an intermediate in leucine catabolism [1] . It has been proposed that in other disorders, 3-methylglutaconic acid derives from aberrant isoprenoid shunting from cytosol to mitochondria via mevalonate pathway or redirection of mitochondrial acetyl CoA toward production of 3MGA due to an increase in the intra-mitochondrial NADH/NAD $^{+}$ ratio resulting from mutation induced impairment in electron transport chain or Kreb cycle function [2] .

Examples of mitochondrial include Barth syndrome, a cardioskeletal myopathy with neutropenia, abnormal mitochondria and MGCA. Barth

syndrome is caused by X-linked recessive mutations in the *TAZ* gene which encodes the mitochondrial membrane localized transacylase involved in the maturation of cardiolipin.

Autosomal recessive mutations in the *OPA3* gene (OMIM: 606580), the mouse ortholog of which encodes a mitochondrial inner membrane protein of unknown function, cause MGCA3 (OMIM: 258501), a neuroopthalmologic syndrome characterized by early-onset bilateral optic atrophy and later-onset spasticity, extrapyramidal dysfunction and cognitive deficit.

MGCA5 (OMIM: 610198) is yet another form of MGCA caused by autosomal recessive mutations in the *DNAJC19* gene (OMIM: 608977) and in addition to increased urinary excretion of 3-methylglutaconic acid, patients present with infancy or childhood onset dilated cardiomyopathy, microcytic anemia, mild muscle weakness and ataxia. Many patients die of cardiac failure. The *DNAJC19* gene encodes the human homolog of the yeast Tim14 which is a part of the Tim23 mitochondrial protein import machinery and hasbeen shown to interact with the mtHsp70 in an ATP-dependent manner to regulate Tim23 function (Davey, 2006).

WE report a distinct type of 3-methylglutaconic aciduria resulting from a mutation in mitochondrial TIMM50 gene in 3 sibs from a consanguineous family. We initially reported these xases in abstract form. Recently two different mutations in mitochondrial TIMM50 gene have been reported in four patients with 3 methylglutaconic aciduria, epilepsy, severe intellectual disability and lactic acidosis.

Subjects

Family 1

Family 1 has three affected sibs of South Asian ancestry with intractable epilepsy, microcephaly, developmental delay, visual deficit spastic quadriplegia. Two affected sibs died unexpectedly when they were visiting families in a remote area of a South Asian country. Metabolic testing had revealed large amount of 3-methylglutaconic acid in urine in all three affected sibs. The patients have a healthy brother and a healthy sister. Mother and father are first cousins. Detailed clinical history, imaging, EEG and metabolic testing were obtained for all affected persons. Full exome sequencing was performed using genomic DNA isolated from one surviving patient, two healthy siblings and both parents.

Patient IV-1. Patient IV-1 was the first born child of the parents and was born at 36 weeks gestation after a normal pregnancy and delivery. Her weight at birth was 1. 99 kg. Her weight, height and head circumference were always below 5 th centile. She also had asthma and frequent episodes of pneumonia presumably due to aspiration, but the family refused G-tube placement. She was severely delayed. She never sat, stand or spoke. She has poor head control, truncal hypotonia but very brisk tendon jerks and sustained clonus. Funduscopy revealed bilateral optic atrophy. She developed seizures at 1 year of age. EEG revealed multifocal spikes arising from both hemispheres. She was treated with phenobarbital and Zonegran but family was noncompliant with medications. She continued to have daily myoclonic jerks. MRI at 2. 5 and 5 years of age showed increased T2 signal in basal ganglia and periventricular white matter, brain atrophy, prominent ventricle,

increased extraxial fluid. Normal liver enzymes and blood count, normal blood and CSF glucose and a serum ammonia of 21. Several serum lactate levels were mildly elevated. Lactate 2. 8, 4. 5 (Pyruvate 0. 23), 5. 4 (normal 0. 7 to 2. 1) Lactate to pyruvate ratio 20: 1. Urine organic acid analysis revealed very high lactic acid, 3-methylglutaconic acid, and 3-methylglutaric acid. Muscle biopsy revealed only scattered atrophic muscle fibers on electron microscopy. Respiratory chain enzyme activities were within normal limits. She died at 7. 5 years of age apparently due to complications from an infection while she was visiting families in a remote area of a South Asian country.

Patient IV-4 was twin A born at 36 weeks gestation after an uncomplicated twin pregnancy. Her weight, height and head circumference were always below 5 th centile. She was severely delayed. She never sat, stand or spoke. She has poor head control, truncal hypotonia but very increased reflexes and spasticity in the limbs. At nine-month-of age, she started to experience several episodes of eye fluttering and body jerking. Her EEG reved slow background, poor sleep architecture and frequent multifocal spike and sharp wave activities coming from both the left and right hemispheres. Her seizures were treated with Zonegran and was poorly controlled but parents refused more aggressive treatment of seizures. Metabolic testing revealed mild elevation of lactate and moderate increase of 3 methylglutaconic, 3 methylglutaric acids in urine. A brain MRI at 11-month-of age revealed diffuse volume loss supratentorially with prominent sulci and extraaxial fluid spaces, mild enlargement of the ventricles and patchy signal abnormalities in the basal ganglia bilaterally, especially involving the caudate nuclei and

putamen. On spectroscopy with voxel placed in the right basal ganglia with short and long TE, there was a lactate peak which inverted on long TE spectrum. Also, the NAA peak was low with NAA to creatinine being 1. 15 on short echo and 1. 29 on long echo spectrum. Also, the choline was elevated with choline/creatine ratio being 1. 00 on short echo and 1. 41 on long echo images. She died at 1. 5 years of age apparently due to complications of an infection while she was visiting families in a remote area of a South Asian country.

Patient IV-5 is a 13 year old female of South Asian ancestry, with 3-methylglutaconic aciduria intractable epilepsy, microcephaly, developmental delay, visual deficit and spastic quadriplegia. She was born at 36 weeks gestation after an uncomplicated twin pregnancy. She was twin B and stayed in NICU for 18 days for feeding issues. Her weight was 1. 4 kg and she was not intubated. Patient first presented with seizures at 3 months of age with eyelid fluter and jerking of extremities. Her initial EEG revealed multifoal spikes. Initial biochemical evaluation revealed normal serum and CSF glucose, normal ammonia and liver enzymes. Serum lactate and CSF lactate 4. 24 mmol were mildly elevated . Lactate was 2. 7. Ammonia 25. Serum amino increased alanine 43. 6 micromol/dl (9. 9-34. 5). Csf lactate 4. 24 mmol. CSF alanine 7 micromol/dl (0. 6 -4. 7). There were also mild elevations of serum and CSF valine, leucine, isoleucine and alanine and lysine. Urine organic analysis revealed moderate increase of 3 methylglutaconic, 3 methylglutaric, glutaric, adipic, suberic, and sebacic acids. MRI of brain at 11 months of age revealed severe atrophic changes involving gray and white matter, predominantly of the cerebrum. Grossly abnormal signal is seen in

the basal ganglia, particularly the caudate nucleus and the putamen with relative sparing of the globus pallidus and thalamus. A recent MRI (at age 13 years) reveals severe but stable atrophic changes of the gray and white matter of the supra and infratentorial brain, stable white matter changes of the putamen, caudate nucleus and periventricular white matter, Scattered diffusion restriction in the retrotrigonal white matter, compatible with active demyelination and atrophic changes of the optic nerves. Her seizures were treated with with Keppra, Lamictal, Zonegran and Onfi. She also receives carnitine. She continues to have brief episodes of whole body stiffening each week, but the family was also not very compliant with medications. Her current EEG shows slow background for age, poorly formed sleep spindles indicatvie of diffuse neuronal dysfunction, frequent multifocal interictal spike and wave suggests increased risk of seizures arising from multiple foci and hypsarrhythmia in sleep . She has failure to thrive despite G-tube feeding. At 12 years of age, G-tube was placed due to history of aspirations. Height, weight and head circumference below 5 th centile. She is severely delayed. She is nonverbal and never learned to sit independently, stand or walk. She recognizes family members, responds to their voice and looks and smiles at them. Her fundoscopy shows mild optic atrophy. She has bilateral esotropia and dysconjugate gaze. She has poor head control and truncal hypotonia, but her limbs are spastic and her tendon reflexes are very brisk.

Family 2

Patient V: 1 was the first son of Caucasian consanguineous parents (IV: 4 and IV: 5) of Eastern European origin. Within the context of an organic acid and amino acid study in young and adult subjects with non-syndromic

developmental delay and intellectual disability, he was investigated at the age of 17 years and presented with a developmental language disorder (involving semantic, syntactic, and pragmatic components of the linguistic system), emotional and communicative problems (fearful, aggressive, and loner), and hyperactivity. On neuropsychological testing he showed a short attention span. The child was born at term after an uneventful pregnancy and his birth weight was 2. 9 kg. At 4 months of age he was affected by myoclonic jerks that were controlled by administration of valproic acid and lamotrigine. Developmental delay was observed starting from the middle of the first year of life, accompanied by decreased muscle tone. He could walk without support only at 6 years. At last medical exam, the patient showed a reduced muscle mass (height 148 cm, Z-score 3. 43; weight 38 kg, Z-score 4. 21; BMI 17. 1 kg/m ^{2} , Z-score 2. 02) and a head circumference of 51 cm (Z-score 2. 76). Due to refusal of parents, no brain imaging studies could be performed. Fundoscopic examination was normal. Laboratory tests, including creatine phosphokinase (CPK), liver enzymes and plasma amino acids, were normal. The profile of urinary organic acids showed a large peak of 3-methylglutaconic acid (113 mmol/mol creatine) and a slightly increased level of 3-methylglutaric acid (17 mmol/mol creatinine).

Patient V: 3 was the younger brother of V: 1, the third child of IV: 4 and IV: 5. He was investigated at the age of 11 years and presented with a clinical phenotype (developmental delay and intellectual and behavioral disorder) similar to that of his brother. The pregnancy and early postnatal course was unremarkable and birth weight was 3. 1 kg. At 3 months he received valproic acid and lamotrigine to control tonic seizures with sudden stiffening

movements of arms and legs. The boy walked independently at 4 years. When he was 9 years, his growing parameters were: height 119 cm (Z-score 2. 47), weight 22 kg (Z-score 1. 91), BMI 15. 5 kg/m ^{2} (Z-score 0. 38), and head circumference 48 cm (Z-score 3. 52). Neuropsychological exam revealed mental retardation and impaired communicative skills, including poor language abilities (few repetitive words with no sentences). Occasionally, the patient is aggressive. Ophthalmologic examination revealed left esotropia. High levels of 3-methylglutaconic acid (155 mmol/mol creatine) were identified in urine, together with smaller amounts of 3-methylglutaric acid (22 mmol/mol creatinine).

Patient V: 5 was the second son of consanguineous parents (IV: 9 and IV: 10) related to those of patients V: 1 and V: 3. The girl was delivered by cesarean section because of growth arrest at 37 week. The neonate showed no external malformations. Birth weight was 2. 1 kg. In the following years, the clinical phenotype was characterized by delayed developmental milestones, nocturnal enuresis, severe cognitive impairment, speech retardation, and lack of communicative skills. Results of the electroencephalogram were normal. No brain imaging data are available. On a few occasions, levels of ammonia and lactic acid were found to be slightly elevated, but these results could not be confirmed by repeated blood analyses. Plasma levels of amino acids are within normal range. Fundoscopic examination was normal up to 7 years, but since then there is evidence of mild bilateral optic atrophy. Urine levels of of 3-methylglutaconic acid and 3-methylglutaric acid were 176 mmol/mol creatine and 29 mmol/mol creatinine, respectively.

DISCUSSION

Deleterious Nature of the *TIMM50* gene alteration:

TIMM50 NM_001001563 c. 1114G> A p. G372S

The p. G372S variant (also known as c. 1114G> A), located in coding exon 9 of the *TIMM50* gene, results from a G to A substitution at nucleotide position 1114. The glycine at codon 372 is replaced by serine, an amino acid with somewhat similar properties. The alteration is not observed in healthy cohorts: Based on data from the NHLBI Exome Sequencing Project (ESP), the *TIMM50* c. 1114G> A alteration was not observed among 6, 503 individuals tested. Allele frequency data for this nucleotide position are not currently available from the 1000 Genomes Project and the alteration is not currently listed in the Database of Single Nucleotide Polymorphisms (dbSNP). Though some variants may appear to be rare due to database-specific ethnic underrepresentation, rare missense alleles commonly exhibit a deleterious effect on protein function (Kryukov, 2007; Tennessen, 2012). The altered amino acid is conserved throughout evolution: The G372 amino acid position is completely conserved in eukaryotes all the way from the yeast Saccharomyces cerevisiae to humans (Mokranjac, 2003). The alteration is predicted deleterious by *in silico* models: The p. G372S alteration is predicted to be probably damaging and deleterious by PolyPhen and SIFT *in silico* analyses, respectively. The amino acid is located in a functionally important protein domain: The p. G372S alteration is located in the conserved C-terminal domain of the Tim50 protein that interacts with the N-terminal domain of the Tim23 protein in the inter membrane space and regulates mitochondrial protein import of presequence-containing

polypeptides (Geissler, 2002; Yamamoto, 2002; Guo, 2004). The alteration cosegregated with disease in the family herein: Co-segregation analysis revealed that this alteration is present in a heterozygous form in the mother, father and brother, and absent in the sister.

Based on the available evidence, the *TIMM50* c. 1114G> A (p. G372S) alteration is classified as a likely pathogenic mutation.

The TIMM50 gene is not currently known to underlie Mendelian disease (aka " clinically novel"). The TIMM50 gene function is consistent with the proband's clinical presentation:

The Translocase of Inner Mitochondrial Membrane 50 (TIMM50) gene (OMIM: 607381) is located on human chromosome 19q13. 2 and consists of 11 exons. It encodes the Tim50 protein, a 353 amino acid 40 kDA homolog of the yeast Tim50 protein that functions as an integral part of the mitochondrial Tim23 protein import machinery by linking protein translocation across the outer and inner mitochondrial membranes. This interaction was confirmed by the coprecipitation of Tim50 with an antibody against Tim23 (Geissler, 2002; Yamamoto, 2002; Guo, 2004). The authors further confirmed that the C-terminal domain of Tim50 is located in the inter-membrane space (IMS) where it stably binds to the segment of Tim23 that spans the IMS and regulates its function.

Nuclear encoded mitochondrial proteins are synthesized in the cytosol and subsequently imported into the mitochondria through the function of translocators, the TOM complex of the outer mitochondrial membrane (OMM), and the Tim23 and Tim22 complexes of the inner mitochondrial

membrane (IMM) (Jensen, 2002). While the Tim22 complex is involved in the transport and insertion of proteins lacking the presequence into the inner membrane, the Tim23 complex is required to process and insert presequence-containing precursor proteins. The IMM generates a proton motive force that is critical for cellular energy synthesis (Stock, 2000) and the permeability barrier of the IMM needs to be maintained during the transport of proteins through the pore-forming Tim23 protein associated with other IMM proteins such as Tim14 (human DNAJC19), Tim17, Tim21, Tim44 and Tim50. Using various yeast IMM protein mutants, Meinecke et al. (2006) demonstrated that tim17 and tim21 mutant mitochondria displayed membrane potential values that were comparable to wild type mitochondria, whereas tim50 mutant mitochondria showed a drastic reduction of the membrane potential. Further functional studies revealed that the Tim23 channel is tightly regulated by Tim50 in its inactive state to maintain the IMM permeability barrier and is opened only when presequence-containing polypeptide chains need to be translocated into the mitochondrial matrix or the inter membrane space (IMS). Loss of Tim50 function in yeast led to cellular growth arrest and reduced cell viability (Mokranjac, 2003). Knockdown to Tim50 expression in cultured human cells using RNA mediated interference resulted in an increase in the release of cytochrome c and apoptosis in response to cell death stimuli (Guo, 2004).

A 50 kDa isoform of the human mitochondrial TIM50, TIM50a, consisting of 456 amino acids has been found to localize in nuclear speckles, specifically in the Cajal bodies, and interact with small nuclear ribonuclear proteins (snRNPs), the coilin protein and the Survival of Motor Neurons (SMN) protein

(Xu, 2005) which has been implicated in Spinal Muscular Atrophy (SMA). The protein sequences of the mitochondrial TIM50 and the nuclear TIM50a are identical with the exception of additional 103 amino acids at the N-terminal of TIM50a that are the result of an alternative translational start sequence. This additional N-terminal sequence in TIM50a is thought to contain a putative nuclear localization sequence that allows the Tim50a isoform to display a nucleus specific localization. Based on their results, Xu et al. hypothesized that Tim50a might be involved in the regulation of snRNP biogenesis and possibly the function of the nuclear SMN protein encoded by the SMN1 gene. One of our patients had mulsle biopsy. Although there were atrophic changes, no neuropthic pattern was seen.

Reference List

(1) Wortmann SB, Kluijtmans LA, Rodenburg RJ et al. 3-Methylglutaconic aciduria–lessons from 50 genes and 977 patients. *J Inherit Metab Dis* 2013; 36: 913-921.

(2) Ikon N, Ryan RO. On the origin of 3-methylglutaconic acid in disorders of mitochondrial energy metabolism. *J Inherit Metab Dis* 2016; 39: 749-756.

Legends

Legend to Figure 1

Five-generations pedigree of the family with mild 3-methylglutaconic aciduria in which the TIMM50 p.(Ile293Thr) was identified. Subjects V: 1, V: 3, and V: 5 (filled symbols) are patients suffering from intellectual disability and increased urinary excretion of 3-methylglutaconic acid. They are born to

consanguineous parents and homozygous for the TIMM50 c. 1011T> C mutation predicting the replacement of isoleucine 293 with threonine in the encoded protein. The mutation was inherited by a common ancestor (either I: 1 or I: 2) and has been identified in the heterozygous state in the clinically and biochemically unaffected subjects III: 3, III: 4, III: 9, IV: 2, IV: 4; IV: 5; IV: 9, IV: 10, and V: 2.