Novel ETFDH mutations in four cases of riboflavin responsive multiple acyl-CoA dehydrogenase deficiency

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\textbf{A B S T R A C T}

Multiple acyl-CoA dehydrogenase deficiency (MADD) is an autosomal recessive disorder of fatty acid, amino acid, and choline metabolism caused by mutations in \textit{EFTA}, \textit{EFTB}, or \textit{ETFDH}. Many MADD patients are responsive to treatment with riboflavin, termed riboflavin-responsive MADD (RR-MADD). Here, we report three novel mutations and one previously reported mutation in \textit{ETFDH} in four RR-MADD patients who presented at various ages, and characterize the corresponding changes in ETF-QO protein structure. Clinicians should consider MADD in the differential diagnosis when patients present with muscle weakness and biochemical abnormalities. Gene testing plays a critical role in confirming the diagnosis of MADD, and may not only prevent patients from invasive testing, but also allow timely initiation of riboflavin treatment. The novel variants in \textit{ETFDH} and the corresponding clinical features reported here enrich the allelic heterogeneity of RR-MADD and provide insight into genotype-phenotype relationships.

\section{1. Introduction}

Multiple acyl-CoA dehydrogenase deficiency (MADD), also known as glutaric aciduria II (GAI, OMIM #231680), is an autosomal recessive disorder of fatty acid, amino acid, and choline metabolism. MADD is highly clinically heterogeneous, and has been categorized into three types: neonatal onset with congenital abnormalities (Type I), neonatal onset without congenital abnormalities (Type II), and late-onset (Type III) [1–3]. MADD results from a defect in either electron transfer flavoprotein (ETF, encoded by the alpha ETF (ETFA) and beta ETF (ETFB) genes) or ETF-ubiquinone oxidoreductase (ETF-QO, encoded by the ETF dehydrogenase (ETFDH) gene) [4, 5].

Types I and II are severe and typically fatal, characterized by nonketotic hypoglycemia, metabolic acidosis, and accumulation and excretion of metabolites, while Type III is milder and more variable, characterized by recurrent episodes of hypoglycemia, metabolic acidosis, vomiting, and muscle weakness during catabolic stress [6, 7]. Many MADD patients, especially late-onset MADD patients, can be effectively treated with riboflavin, termed riboflavin-responsive MADD (RR-MADD). Previously, it has been reported that \textit{ETFDH} mutations are the major cause of RR-MADD [8, 9]. Here, we report four additional RR-MADD patients from three unrelated families who presented at various ages and carry three novel mutations and one previously reported mutation in \textit{ETFDH}. In addition, we predict changes in the ETF-QO protein structure due to these mutations.

\section{2. Materials and methods}

\subsection{2.1. Patients}

The four patients are from southern China, and were clinically diagnosed with MADD. Informed consent was obtained, and this study...
creased muscle enzymes. His clinical symptoms improved for 20 days.

hypoglycemia, metabolic acidosis, electrolyte disturbances, and in-
gradually recovered to normal levels.

0.2 mg) three times a day, and his muscle strength and lab values
listed in Table 1.

3.1. Clinical presentations

2GMH) by homology modeling (Swiss-Model).

were identified, Patient 2 and Patient 3 were identified in ETF-B and ETF-A, respectively. Whole exome sequencing (WES) was
performed for the other two patients. Whole exome sequencing, exome
enrichment, and Sanger sequencing were used for confirmation of candidate variants. Deletions variants were
identified in ETF-B and ETF-A, and the Genome Analysis Toolkit (GATK) and the Genome Analysis Toolkit (GATK) were performed using Illumina
HiSeq2000 (Illumina, CA, USA) according to the manu-
ufacturer's protocols, and sequencing was performed on an Illumina
Redwood City, CA, USA). SIFT, Polyphen2, and MutationTaster were
used for in silico analysis of candidate variants. Deleterious variants
were confirmed by Sanger sequencing.

2.3. Structure prediction of ETF:QO

was approved by the local ethics committee.

was performed using Ingenuity Variant Analysis pipeline (Ingenuity,
and the Genome Analysis Toolkit. Variant interpretation of WES data
were performed for the other two patients. Sequence capture, enrichment,
and the Genome Analysis Toolkit (GRCh37) were performed using BWA
HiSeq2000 (Illumina). Sequence alignment and variant calling against
the reference human genome (GRCh37) were performed using BWA
Enrichment kit (Illumina, San Diego, CA, USA) according to the man-
ufacturer's protocols, and sequencing was performed on an Illumina
Redwood City, CA, USA). SIFT, Polyphen2, and MutationTaster were
used for in silico analysis of candidate variants. Deleterious variants
were confirmed by Sanger sequencing.

Patient 4, a 30-day old male infant, presented with non-ketotic
acidemia. She has had intermittent mild symptoms, which her parents believed
to be in her sleep. Patien 2, a 2-year old female, had a history of neuromuscular disease. She presented with pneumonia and
history of dislocation in the right shoulder. She was intermittently treated (her parents stop giving her
the medication), and she is now years old and remains symptom-free. Patient 1 was tested at 7 years old following her sibling's diagnosis.

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Table 1

| No. | Sex | Onset | Blood acylarnitine spectrum (μmol/L) | Urine organic acids spectrum | CK (U/L) | LDH (U/L) | AST (U/L) | Genotype |
|-----|-----|-------|-------------------------------------|-----------------------------|-------|---------|---------|----------|
| 1   | F   | 7 yo  | ↑ C2 (6.16), C3 (0.47) ↑ C4 (1.18), C5 (0.56), C6 (1.06), C8 (1.47), C10 (1.86), C10:1 (0.55), C12 (0.60), C14:1 (0.57) | ↑ Ethylmalonic acid (8.48), decanedioic acid (54.59) | 193   | 234     | 34      | c.524G > A (p.R175H), c.229G > A (p.G77S) |
| 2   | M   | 10 do | ↑ C4 (1.03), C5 (0.58), CSDC (0.33), C6 (0.96), C8 (1.50), C10D (0.23), C10 (2.11), C10:1 (0.36), C12 (2.43), C12:1 (0.45), C14 (2.49), C14:1 (1.53), C16 (6.39), C16:1 (1.47) | ↑ 3-Hydroxybutyric acid (29.22) | 235   | 331     | 82      | c.524G > A (p.R175H), c.229G > A (p.G77S) |
| 3   | M   | 27 yo | ↑ C3 (5.37), C3 (0.42) ↑ C4 (1.16), CSDC (0.11), C20:1 (0.51), C14:1 (1.13), C14:1 (1.18), C16 (4.16), C14:1 / C8:1 (32.10), C16/C3 (9.92) | ↑ Lactate-2 (34.29), Glycolic acid-2 (14.44), Oxalic acid-2 (4.42), 2-Hydroxybutyric acid-2 (4.58), 3-Hydroxypropionic acid-2 (4.43), Pyruvate-OX-2 (58.29), 2-Hydroxyisovalerate-2 (17.98) | 1318  | 1024    | 557     | c.524G > A (p.R175H), c.1450T > C (p.W484R) |
| 4   | M   | 30 do | ↑ C3 (5.37), C3 (0.42) ↑ C4 (1.16), CSDC (0.11), C20:1 (0.51), C14:1 (1.13), C14:1 (1.18), C16 (4.16), C14:1 / C8:1 (32.10), C16/C3 (9.92) | ↑ Ethylmalonic acid (10.33), 3-Hydroxybutyric acid (7.03), 4-Hydroxyphenylacetic acid (28.34) | 976   | 853     | 323     | c.1157G > A (p.G386D), c.1450T > C (p.W484R) |

↑ above normal level; ↓ below normal level; yo: years old; do: days old.

Upper limit of normal: CK 200 U/L, LDH 245 U/L, and AST 40 U/L.
after riboflavin treatment (treatment details unknown). At 3 months old, Patient 4 was hospitalized for pneumonia, and passed away due to sepsis. The sister of Patient 4 (genotype unknown) passed away at 3 months old due to encephalitis.

3.2. Mutation analysis

Sequencing studies identified four missense mutations in the three families, three of which are novel (hg19; Chr4:159603400 G > A, c.229G > A, c.1157G > A, c.1450T > C) detected in this study marked with asterisks. a) Schematic of the human ETFDH gene structure with the three novel mutations (c.229G > A, c.1157G > A, c.1450T > C) detected in this study marked with asterisks. b) Segregation of heterozygous missense mutations in Family 1 (F1), Family 2 (F2) and Family 3 (F3). c) Population frequencies and in silico predictions for detected mutations. d) Depiction of the DNA and protein changes, with the mutations marked with red arrows and the corresponding amino acid changes in red. e) The four missense mutations are conserved across species. D: Damaging. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Protein structure analysis

ETF-QO, encoded by ETFDH, is integrated in the inner mitochondrial membrane and its crystal structure is comprised of three domains, as depicted in Fig. 2: FAD domain (FAD shown in green), 4Fe4S cluster domain (4Fe4S shown in magenta), and UQ-binding domain (UQ shown in red). The four variants are shown as cyan spheres (Fig. 2a). R175, G77, and G386 are located near the FAD binding site, while W484 is located near the UQ binding site. The mutants R175H, G77S, and G386D likely affect the binding affinity of ETFDH to FAD.
Therefore, these variants may reduce the activity of ETFDH by decreasing the affinity of ETFDH binding to FAD or UQ10.

4. Discussion and conclusions

In this study, we report three novel compound heterozygous mutations in ETFDH in four patients with RR-MADD from three unrelated families. The diagnosis of MADD was primarily based on biochemical data (increased levels of acylcarnitines and urine organic acids), and confirmed by ETFDH mutation analysis. Although RR-MADD is a treatable disease, it is rare and its diagnosis is difficult due to high clinical heterogeneity. MADD patients often present with fluctuating muscle weakness, vomiting, hypoglycemia, metabolic acidosis, encephalopathy, and hepatopathy [12–14]. However, many muscle disorders (such as inflammatory myopathies, metabolic myopathy, and progressive muscular dystrophy) also present with muscle weakness. Thus, MADD may be misdiagnosed as a different type of lipid storage myopathy, a glycogen storage disease, progressive muscular dystrophy, or other muscle disease [15]. Muscle biopsy has traditionally been diagnostically important to differentiate MADD from other muscle diseases, as muscle biopsy showing accumulation of lipid droplets in muscle fibers in combination with the biochemical pattern of acylcarnitines and urine organic acids can confirm the MADD diagnosis [16].

Currently, gene testing plays an important role in MADD diagnosis, and may prevent more invasive diagnostic testing, such as muscle biopsy. Given the current knowledge of the genetic etiology underlying MADD, we suggest a stepwise approach for patients suspected to have MADD involving targeted sequencing of ETFA, ETFB, and ETFDH followed by WES if targeted sequencing is negative. In addition to cost effectiveness, this approach would limit incidental findings such as of variants of unknown significance, which are difficult to interpret and may cause anxiety for the patients and their families. For example, a recent report of a patient with MADD used WES to identify a compound heterozygous mutation in ETFDH as well as a predicted deleterious novel variant in ACOT11, which was of unclear relevance to the phenotype [17].

Early diagnosis is critical because timely riboflavin treatment is effective in many cases. Riboflavin, the precursor of the coenzyme FAD, has been reported to act as a molecular chaperone that promotes the in vitro folding and steady state levels of misfolded ETF-QO proteins in early stages and stabilizes folding intermediates or membrane-inserted proteins in later stages [18, 19]. Riboflavin treatment was given to all four patients in this study, and they showed dramatic improvement in clinical symptoms after riboflavin treatment. Patient 4 was re-hospitalized for pneumonia, and was treated late in his disease course. He suffered from severe liver injury, which was considered to be irreversible, and led to the family's decision to withdraw support.

Although over 80 mutations in ETFDH have been reported worldwide, the same mutation is rarely found in different populations [20]. Hot spot mutations have been identified in Asian populations, with c.250G > A (p.A84T) the most common mutation in southern China [21], and c.770A > G (p.G362R) and c.1227A > C (p.L409F) the most common mutations in northern China [9, 22–24]. Indeed, ETFDH mutations may be ethno-specific. In the four RR-MADD patients from southern China in this study, we identified three novel mutations and one previously reported mutation in ETFDH [11]. Of note, Patients 1 and 2 carried the same mutations but presented at different times and with different clinical manifestations, suggesting the role of modifier genes or the environment. Further studies are needed to elucidate these differences.

ETFDH mutations can be classified into two groups: mutations affecting protein folding and assembly or mutations affecting enzymatic activity [25]. Three of the identified mutations in this study are located in the FAD binding domain (p.Arg175His, p.Gly77Ser, and p.Gly386Asp), and one is located in the UQ binding domain (p.Trp484Arg). Most of the previously reported ETFDH mutations are
located in the FAD binding domain, suggesting that this region is a sensitive functional hot spot [26]. Riboflavin treatment has been suggested to promote FAD binding to ETF-QO mutants with variants in the FAD domain by affecting the kinetics and/or thermodynamics to stabilize the mutant conformations [27].

In summary, we report four variants including three novel likely pathogenic mutations in ETFDH, enriching the allelic heterogeneity of MADD. A high index of suspicion for MADD is necessary if a patient presents with symptoms including muscle weakness, and it should be included in the differential diagnosis. Gene analysis is critical to confirm the diagnosis of MADD, and may prevent invasive testing, such as muscle biopsy, and allow timely initiation of effective therapy to alleviate symptoms.

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