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Novel mutations in *ETFDH* gene in Chinese patients with riboflavin-responsive multiple acyl-CoA dehydrogenase deficiency

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ABSTRACT

Background: Multiple acyl-CoA dehydrogenase deficiency (MADD, OMIM 231680) or glutaric aciduria type II (GAII) is an inherited autosomal recessive disease affecting fatty acid, amino acid and choline metabolism, due to mutations in one of three genes namely, electron transfer flavoprotein alpha-subunit, *ETFA* (OMIM 608053), electron transfer flavoprotein β-subunit, *ETFB* (OMIM 130410) and electron transfer flavoprotein dehydrogenase, *ETFDH* (OMIM 231675). Some MADD patients are responsive to riboflavin treatment with an excellent prognosis. Recently, mutations in *ETFDH* were found to be responsible for all riboflavin-responsive MADD patients. In this study, we present the clinical features and molecular studies of 2 Chinese families with riboflavin-responsive MADD.

Methods: Genomic DNA was extracted from peripheral blood samples or skin fibroblast cultures from the patients and normal controls. The thirteen exons of *ETFDH* were amplified by PCR. PCR products were sequenced in both forward and reverse directions. To rule out mutations in other genes, phenotype segregation was studied in the families by microsatellite markers in the proximity of the 3 genes, *ETFA*, *ETFB* and *ETFDH*.

Results: Four novel mutations in *ETFDH* were detected in the 2 families. In family 1, a frame shift mutation, c.1355delG which introduced a premature-termination codon (PTC), I454X in exon 11 of *ETFDH* was found. Another mutation was a c.250G>A transition in exon 3 of *ETFDH*, A84T. In family 2, two novel missense mutations were identified, P137S, in exon 4 and G467R in exon 11. No carrier of these four mutations was identified from about 150 alleles of healthy Chinese control subjects.

Conclusions: Four novel mutations (3 missenses and 1 deletion) in *ETFDH* were found in Chinese families that presented with riboflavin-responsive MADD, which further expands the list of mutations found in patients with riboflavin-responsive MADD. Furthermore, we illustrated the utility of phenotype–genotype segregation in MADD families to prioritize genes for sequencing or to rule out the presence of disease causing mutation in other genes in MADD and other diseases caused by multiple genes.

1. Introduction

Multiple acyl-CoA dehydrogenase deficiency (MADD, OMIM 231680) or glutaric aciduria type II (GAII) is an autosomal recessive disease causing defects in mitochondrial electron transfer and metabolism of fatty acid, amino acid and choline. The disease is characterized by the excretion of excessive amount of aliphatic mono- and dicarboxylic acids, sarcosine and glycine conjugates. Patients may present clinically as one of three different forms with variable degrees of clinical severity. They are type I, the neonatal-onset form with congenital anomalies; type II, a neonatal-onset form without congenital anomalies, and type III, a late-onset form [1]. Type I patients usually present in infancy with polycystic kidneys, severe nonketotic hypoglycemia, metabolic acidosis, excretion of large amounts of fatty acid- and amino acid-derived metabolites and may die in the newborn period. Type II patients are similar to type I patients except that congenital anomalies are not present. In contrast, the age at presentation of type III MADD is later and symptoms are highly variable. These patients may present with recurrent episodes of lethargy, vomiting, hypoglycemia, metabolic acidosis, and hepatomegaly particularly during metabolic stress. Muscle pain, weakness, and lipid-storage myopathy may also be present. Characteristic organic aciduria may only be detectable during periods of illness or catabolic stress. In most cases, an altered function of

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either electron transfer flavoprotein (*ETF*) or its dehydrogenase (*ETFDH*) has been demonstrated [2–4].

ETF is a heterodimer of 30-kD alpha-subunits (*ETFA*) and 28-kD β-subunits (*ETFB*) [5], whereas *ETFDH* exists as a monomer [6]. The 3-D structure of *ETFDH* has been reported [7]. This enzyme complex encoded by 3 genes, is required for electron transfer from more than 9 flavin-containing dehydrogenases to the main respiratory chain in the mitochondria.

Most MADD patients may carry mutations in any of the 3 genes [1,4,8]. Interestingly, Olsen et al. found that mutations were confined to *ETFDH* in all riboflavin-responsive MADD patients from 11 pedigrees [9]. Also, Gempel et al. reported that several late-onset GAII patients presented with myopathy and a secondary CoQ10 deficiency were responsive to riboflavin treatment. All of them were found to have mutations in *ETFDH* [10]. The exact mechanism of how riboflavin can ameliorate the symptoms of riboflavin-responsive MADD is still unknown. However, decreased activity of *ETFDH* together with a secondary perturbation of mitochondrial flavin and flavoprotein homeostasis might be implicated in these patients. Indeed, several patients with riboflavin-responsive MADD were found to have decreased levels of flavins in mitochondria and mitochondrial respiratory chain complexes dysfunction [9,11,12]. Previous studies on the occurrences of IMD in Hong Kong Chinese have revealed that MADD was among the recurrent diagnoses of the group of FAOD, in addition to primary carnitine deficiency and carnitine-translocase deficiency. This hospital has diagnosed four unrelated southern Chinese families of MADD in the past years [13,14]. Two families are classified as riboflavin-responsive MADD. All patients in these families presented with typical clinical and biochemical features of this fatty acid oxidation defect including characteristic urine organic acid and acylglycine profiles. So far, no mutation data on Chinese riboflavin-responsive MADD patients can be found in the literatures. In this study, we present the clinical features and molecular studies of three Chinese patients from these 2 families with riboflavin-responsive MADD.

2. Patients and clinical history

2.1. Family 1, case 1

A 20-year old female Chinese [case 6.2] presented to the hospital at 7 years with muscle weakness for 3–4 years and was noted to have worsened in last 2 months. At presentation, her urine showed typical organic acid pattern characteristics of MADD including glutaric, 2-OH glutaric, ethylmalonic, acetoacetic, 3-OH butyric, saturated and unsaturated dicarboxylic acids, 3-OH saturated and unsaturated dicarboxylic acids and glycine conjugates. Subsequently, she had intermittent abnormal urine organic acid results and usually associated with muscle weakness. She was put on 100 mg riboflavin daily and followed up regularly. Her elder sister died of a Reye's like syndrome in childhood.

At the age of 14 y, the patient stopped taking riboflavin by herself for 4 months. She was admitted to the hospital because of poor appetite, weight loss, lethargy and proximal muscle weakness. Her urine organic acid pattern on admission was consistent with MADD and the patient was advised to continue the riboflavin treatment. After treatment for 3 days, muscle power and appetite improved. Blood samples were collected with written consents from the patient, her mother [case 5.2] and a normal sibling [case 7.2] for genetic testing for MADD.

2.2. Family 2, case 2 & 3

A 4-year old Chinese girl [case 18] was presented to A&E with hypoglycemic convulsion. She was all along well until 10 months of age and was noted to have generalized floppiness following a chest infection. Other aspects of development were normal. Ten days before the current admission, she was admitted to the hospital with convulsion with preceding history of feeling unwell for 2 days. On admission, she was drowsy and had generalized hypotonia and mild hepatomegaly. Laboratory investigations revealed nonketotic hypoglycemia with increased plasma creatine kinase level. Urine organic acid profile was consistent with a diagnosis of MADD. On day 2, her condition deteriorated and required intubation. Her consciousness and peripheral muscle power improved after 2 weeks of treatment. She was treated with riboflavin 100 mg daily and discharged 2 months after admission with normal muscle power and no demonstrable neurological sequelae. Her younger sister [case 19] was 18 month-old when the elder sister was diagnosed. She was picked up by family screening using urine organic acid analysis. She has been put on riboflavin (100 mg/day) and carnitine (50–60 mg/kg/day) since 18 months of age and has been asymptomatic. Both patients now 13 and 16 y, respectively, have been maintained on riboflavin (100 mg/daily) and carnitine (50–60 mg/kg/day) therapy, and regular dietary advice and are physically well.

3. Materials and methods

Genomic DNA was extracted from peripheral blood samples or skin fibroblast cultures by Amersham Biosciences GFX genomic blood DNA purification kit (GE Healthcare, Buckinghamshire, England, UK).

3.1. Determination of familial segregation of the three loci by microsatellites

A pair of microsatellite markers was chosen in the proximity at each of the 3 genes. These markers except D15S215, all had heterozygosity above 0.7 and were flanking the genes of interest. In Supplementary data <Table 1> shows the forward and reverse primers for PCR amplification of respective microsatellites. Genotypes of family members of the two families were determined by DNA fragment length analysis on the ABI Prism 3100 using the GeneScan programme.

3.2. PCR and direct DNA sequencing of the ETFDH gene

The thirteen exons of *ETFDH* gene were amplified by PCR. The sequences of the forward and reverse PCR primers were based on those reported by Olsen et al. [8] with modifications. PCR products were sequenced in both forward and reverse directions. PCR was performed in a PTC-200 Peltier Thermal Cycler (MJ Research, St. Bruno, Quebec, Canada). The PCR products were purified with Montage PCR Centrifugal Filter Devices (Millipore, Billerica, MA). The sequencing PCR products were purified on a column containing 20% Sephadex G50 (GE Healthcare, Buckinghamshire, England, UK). BigDye Terminators DNA Sequencing Kit and ABI PRISM 3100 automated DNA Sequencer for DNA sequencing and reagents for fragment sizes analysis were from Applied Biosystems (Foster City, CA).

3.3. PCR-restriction fragment length polymorphism (RFLP) analysis for allele frequencies of novel mutation

The frequencies of mutations found in the *ETFDH* gene were determined by RFLP analysis in the general population of southern Chinese, and a consecutive collection of 75 anonymous umbilical cord blood samples was used as control to screen for such mutations. The restriction enzymes and primers to be used were determined by whether the cutting site of a restriction enzyme was altered by the mutation using the Webcutter 2.0 (freeware by Max Heiman of Yale University). Protocols for screening of alleles in *ETFDH* gene were shown in Supplementary data <Table 2>. Restriction enzymes used in the study were from New England Biolabs Inc. (Ipswich, MA).

4. Results

4.1. Familial segregation of the three loci by microsatellites

In family 1, the haplotype at *ETFDH* locus (142 bp and 209 bp fragments) was shared by the patient and her mother, whereas, another haplotype (142 bp and 193 bp fragments) was shared by the patient and her younger unaffected brother which was derived from her father (Fig. 1a). In family 2, it was clear that disease phenotype only segregated with D4S413 and D4S2982 at the *ETFDH* loci in chromosome 4q. The *ETFDH* haplotype (140 bp–193 bp) was present in all the family members tested, whereas, the two affected siblings, cases [18] and [19] shared an additional *ETFDH* haplotype of (148 bp–209 bp), which was consistent with paternal origin (Fig. 1b). On the

other hand, the two other genes (*ETFA*, *ETFB*) showed no identical haplotypes between the 2 affected family members. In contrast, phenotype segregation cannot be examined in family 1 as there was only one affected member.

4.2. Mutation analysis of ETFDH of the two families

The results of mutation analysis of *ETFDH* of the two families are summarized in Table 1. We identified four novel mutations in *ETFDH* in the two families. As shown in Supplementary Fig. 2a and b, case [6.2] caused a single base-pair deletion leading to frame shifts, c.1355delG in exon 11 of *ETFDH*. The patient's mother, case [5.2] was a carrier of the deletion mutation. Another mutation A84T, a c.250G>A transition in exon 3 of *ETFDH* was also found in the elder brother, case [7.2]. The father was not available for testing. The two mutations were shown to be derived from separate alleles marked by the two microsatellites — D4S413 and D4S2982. The carrier frequencies of the two mutations were determined by RFLP with PvuII and Hpy188I digestion, respectively. No carrier of these two mutations was identified from 77 (154 alleles) and 75 samples (150 alleles) of Chinese control subjects.

As shown in Supplementary Fig. 3a and b, 2 novel mutations in *ETFDH* were shared by both affected sibling pair in family 2. The missense mutation, P137S, a c.409C>T transition in exon 4, was found in the affected siblings but not present in the mother, case [16] and the unaffected brother, case [17]. Another missense mutation, G467R, c.1400G>C transversion also in exon 11, was carried by the mother and all siblings. The father was not available for testing. These two mutations were confirmed to be located in separate alleles marked by the 2 microsatellites — D4S413 and D4S2982. The haplotype (140 bp–193 bp) was present in all the family members tested confirming that G467R was derived from the mother. Both affected siblings shared another identical haplotype (148 bp–209 bp), which is of paternal origin and segregated with P137S. The carrier frequencies of the two

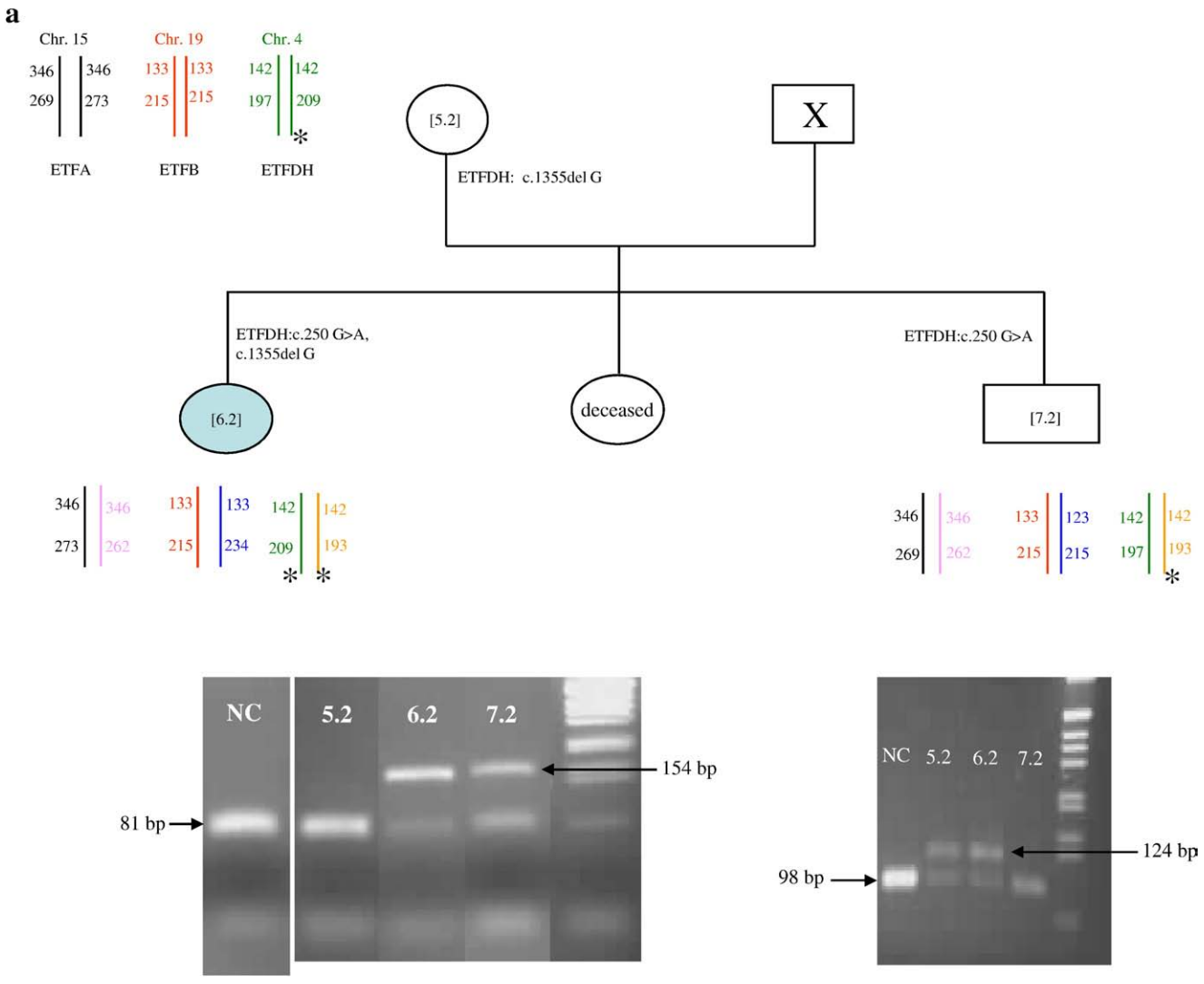

Fig. 1. Genotypes of family members of the 2 families. The family trees, genotypes and microsatellite haplotypes are shown for the 2 families. 2 microsatellites were examined in each of the 3 gene loci, *ETFA, ETFB, ETFDH*. Haplotypes of these 3 loci are shown for each individual. (1a) In family 1, the patient, case [6.2] is compound heterozygous of I454X and A84T mutation in *ETFDH*, the mother, case [5.2] is a carrier of I454X and the unaffected brother, case [7.2] is a carrier of A84T. The restriction digestion patterns for I454X and A84T of a control and the family members are shown in the lower right and lower panels, respectively. For I454X, wild type allele yields a 98 bp fragment, while the mother and the patient have both 98 bp and 124 bp (mutant) fragments. For A84T, control and mother shows an 81 bp fragment, and the patient and the unaffected brother are both carriers of 81 bp and 154 bp (mutant) fragments. (1b) In family 2, the mother, case [16], two affected sisters, cases [18] and [19], and the unaffected brother, case [17] have a mutation in *ETFDH*, G467R. Another mutation P137S is present in both sisters. The restriction digestion patterns for G467R and P137S of a control and the family members are shown in the lower right and left panels, respectively. For G467R, control (NC) has a 186 bp fragment, and all family members carry both 186 bp and 162 bp (mutant) fragments. For P137S, NC, the mother and the brother do not have this mutation and have a wild type 193 bp fragment only. Both affected sisters carry an additional mutant fragment of 163 bp confirming that they are compound heterozygous of G467R and P137S. Microsatellite haplotypes marked with asterisks indicate the chromosome in which disease mutations were found.

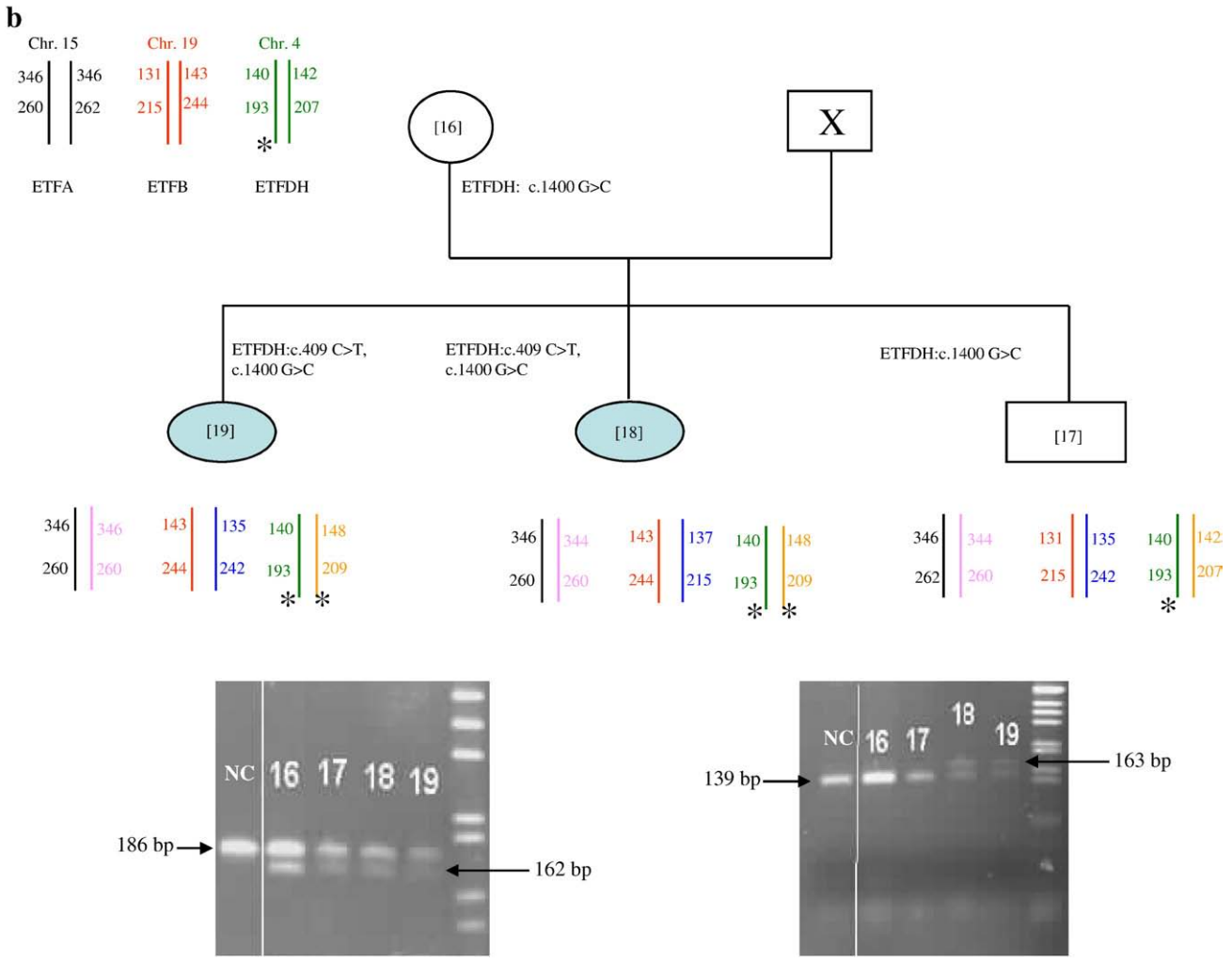

Fig. 1 (*continued*).

mutations were determined by RFLP using enzyme AvaII and RsaI, respectively. No carrier of these 2 mutations was identified from 75 samples (150 alleles) of Chinese control subjects.

4.3. Screening of the new missense polymorphism in the Chinese population

The allele frequencies of the previously recognized polymorphisms c.92T>C (I31T) in exon 3 of *ETFDH* in local population were 0.027. The I31T change is considered to be neutral as it resides in an uncharged residue in the leader sequence of the peptide. Interestingly, the allele frequency of I31T in a US population was much more prevalent with allele frequency of 0.27 [15] and was 0.13 in a Danish population [9].

Table 1
Mutations/polymorphisms detected in southern Chinese.

Case	Gene	Allele 1 codon/ mutation	Exon	Allelic frequency	Allele 2 codon/ mutation	Exon	Allelic frequency
[6.2]	*ETFDH*	I454X/ c.1355delG	11	0/154	A84T/ c.250G>A	3	0/150
[18] & [19]	*ETFDH*	P137S/ c.409C>T	4	0/150	G467R/ c.1400G>C	11	0/150
	ETFDH	I31T/c.92T>C	2	4/148			

All mutations found in this study are novel mutations not reported in the literature.

5. Discussion

Riboflavin-responsive MADD was first described by Gregersen et al. [16] and subsequently by Rhead et al. [11]. Biochemically, it was suggested that the primary defect was caused by mitochondrial electron transfer by flavin metabolism. Early studies showed that some defects in production of co-factors was implicated, such as decreased levels of FMN and FAD within mitochondria were demonstrated in fibroblasts and skeletal muscles of these patients [11,12,16]. However, these proposed defects were not supported by recent molecular study in patients with riboflavin-responsive MADD [9,10]. Instead, Olsen et al. showed that the molecular defects of riboflavin-responsive MADD were caused by mutations in one of the electron transfer flavoproteins, the *ETFDH* gene [9]. They studied 15 patients in 11 pedigrees with the disorder, and mutations were found only in the *ETFDH* in all cases, though they also sequenced the other 2 *ETF* protein subunits. Furthermore, genotype–phenotype correlation suggested that specific mild riboflavin-responsive *ETFDH* mutations might be responsible for the mild phenotype and most patients carried at least one mild mutation. These mutations included c.1448C>T (P483L), c.244T>C (S82P), c.1367C>T (P456L), c.1351G>T (V451L) and c.508G>T (G170C) [9], and c.1768A>G (K590E), c.1130T>C (L377P) [10]. Based on the crystallized structure of porcine *ETFDH*, it was revealed that most of these mutations were located in the ubiquinone-binding domain, except S82P in the FAD binding domain and K590E in the iron–sulfur cluster domain. Interestingly, none of these missense mutations interact directly with the co-factors [7].

MADD may be caused by mutations in any of the three genes forming the flavin dependent electron transfer pathway. Extensive effort of mutation analysis is needed to cover all three genes. It is preferable to study the phenotype–genotype segregation in families and it helps to prioritize or rule out loci for the sequencing step. There are 2 scenarios in which haplotype segregation will contribute to identify the gene locus carrying mutations. First, as illustrated in family 2, segregation of two identical-by-descent haplotypes among affected family members will pin-point the disease causing locus in such family. On the other hand, segregation of two identical-by-descent haplotypes between affected and unaffected sibs will potentially exclude that disease locus. This scenario may be found useful when there only one affected case together with other healthy sibs.

In this study, the case [6.2] presented at 7 years as mild-late-onset MADD and she improved clinically by riboflavin treatment. She has one deletion mutation I454X in exon 11 which produces a truncated protein and one missense mutation A84T in exon 3 of *ETFDH*. The A84T which is located very close to one of the mild mutations, S82P, was described in Olsen's study [9]. This mutation likely accounts for the mild phenotype and response to riboflavin. In contrast, case [18] presented earlier at 10 months of age and was more severe than case [6.2] with multiple episodes of nonketotic hypoglycemia and convulsions requiring hospitalization. On the other hand, her younger sister, case [19] was clinically well at the age of 18 months and was only diagnosed by family screening after diagnosis of the proband. They are compound heterozygotes for the missense mutations, P137S and G467R. Both mutations are novel. A few mutations close to these positions have been reported including L138R [17], P456L and P456T [9]. Among these, only G467R is located at the ubiquinone-binding pocket which surrounds the second through the fifth isoprene units of the ubiquinone. This region is normally comprised of hydrophobic residues, including F147, V158, G467, M468, T471 and G472 [7]. The change from a hydrophobic residue (glycine) to a positive charge polar residue (arginine) may have great impact on the electron transfer from ubiquinone to complex III. Indeed, a patient homozygous for a nearby mutation, G472R, presented 24 h after birth with metabolic acidosis, hypoglycemia, hypotonia, pronounced fatty liver and died at 3 days [8]. Therefore, it is likely that G467R is a severe deleterious mutation. On the other hand, the P137S is located in the FAD binding domain which comprises residues P50 to N139. It is uncertain whether a change from proline to serine is responsible for the riboflavin-responsiveness.

In summary, we report 3 patients in 2 southern Chinese families that presented with riboflavin-responsive MADD using a new mutation detection approach to reduce the sequencing test. Four novel mutations (3 missenses and 1 deletion) in *ETFDH* gene were found in this study which further expands the list of mutations found in patients with riboflavin-responsive MADD reported by Olsen et al. [9] and Gempel et al. [10].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cca.2009.02.015.

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