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ORIGINAL ARTICLE

Riboflavin-responsive multiple Acyl-CoA dehydrogenation deficiency in 13 cases, and a literature review in mainland Chinese patients

Min Zhu[1], Xuan Zhu[1], Xueliang Qi[2], Ding Weijiang[2], Yajing Yu[1], Hui Wan[1] and Daojun Hong[1]

Multiple Acyl-CoA dehydrogenation deficiency (MADD) is an autosomal recessive disorder of fatty acid oxidation and amino-acid metabolism. Most patients with late-onset MADD are well responsive to treatment with riboflavin, which is also termed as riboflavin-responsive MADD (RR-MADD). In this study, we summarized the clinical profiles and genetic features of 13 Chinese patients with RR-MADD and reanalyzed the existing data on RR-MADD patients in Mainland China. In a cohort comprising 13 patients, all were seen to present with severe muscular symptoms occasionally accompanied with mild involvements of extramuscular organs. A total of 18 mutations (13 reported and 5 novel) of the *ETFDH* gene were identified in this series of patients. Exon deletion/duplication was not found in all patients. ETF:QO expression from the muscle specimens was significantly decreased in all patients. At the time of this study the total number of RR-MADD cases had reached 148 in Mainland China since 2009. The muscle symptoms in Mainland China were similar to those in other regions. However, the common extramuscular symptoms were fatty liver and recurrent vomiting in mainland Chinese patients rather than encephalopathy found in Caucasian patients. A total of 68 mutations had been identified in 148 patients with RR-MADD. The c.250G>A had a high mutation frequency in Southern China, whereas c.770A>G and c.1227A>C were more geographically widespread hot spot mutations in Mainland China.
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Keywords: electron transfer flavoprotein dehydrogenase; genetic mutation; hot spot mutation; multiple Acyl-CoA dehydrogenation deficiency; riboflavin

INTRODUCTION

Multiple Acyl-CoA dehydrogenation deficiency (MADD) is an autosomal recessive inherited disorder of fatty acid, amino acid and choline metabolism caused by defects of electron transfer flavoprotein (ETF, encoded by the alpha ETF (*ETFA*) and beta ETF (*ETFB*) gene) or ETF-ubiquinone oxidoreductase (ETF:QO, encoded by the ETF dehydrogenase (*ETFDH*) gene).[1,2] According to the onset age, MADD has considerable clinical variations, from neonatal lethal forms to mild late-onset forms. Neonatal-onset MADD is usually lethal and these patients often die from severe acidosis, nonketotic hypoglycemia and cardiomyopathy during the neonatal period.[3] Symptoms of late-onset MADD are mild and variable, characterized by episodes of encephalopathy, hypoglycemia, vomiting and muscle weakness, usually triggered by metabolic stress.[1,4] Most patients with late-onset MADD can be totally or partly cured on treatment with riboflavin; hence, this clinical phenotype was called riboflavin-responsive MADD (RR-MADD).[1,5] Most cases of RR-MADD present with fluctuating muscle weakness, exercise intolerance, myalgia and dramatic riboflavin responsiveness; symptoms of cardiac or gastrointestinal disorders are occasionally observed in some patients.[6]

RR-MADD has been reported in Europe, in countries such as Denmark,[1] Germany,[7] France[8] and Italy.[9] However, in Asian countries, RR-MADD has been widely and largely found in Mainland China,[4,6,10–14] and a few cases in Taiwan,[15,16] Hong Kong,[17] Japan[2,18] and Thailand.[19] Furthermore, almost all cases of RR-MADD are caused by mutations of the *ETFDH* gene in Mainland China. Although the number of RR-MADD cases in Mainland China is several-fold higher than that in other regions, there is no systematic literature published on the clinical features and gene mutations found in mainland Chinese patients with RR-MADD. Herein, we report 13 cases with RR-MADD and reanalyze the existing data on RR-MADD patients in Mainland China.

MATERIALS AND METHODS

Subjects

A total of 13 patients with RR-MADD were recruited from 143 patients who had undergone muscle biopsy at our neuromuscular disorder center

[1]Department of Neurology, The First Affiliated Hospital of Nanchang University, Nanchang, P.R. China and [2]Department of Neurology, The Second Affiliated Hospital of Nanchang University, Nanchang, P.R. China
Correspondence: Dr D Hong, Department of Neurology, The First Affiliated Hospital of Nanchang University, Yong Wai Zheng Street 17#, Nanchang 330006, P.R. China.
E-mail: hongdaojun@hotmail.com
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(serving patients for more than 14 years) between December 2011 and August 2013. All subjects from Jiangxi province (geographically located in southern China) were examined and interviewed by at least two neurologists (Zhu M., Qi X., Wan H. and Hong D.). The diagnosis of RR-MADD was based on muscle pathological findings, including small, round or irregular vacuoles on hematoxylin and eosin (HE) stain and numerous lipid droplets accumulating in the vacuoles on oil red O (ORO) staining. In addition, all patients experienced complete symptom improvement after treatment with riboflavin within 1 month. The follow-up period ranged from 2 to 20 months (9.2 ± 3.5 months). Six normal muscle samples were obtained from adult patients who had undergone surgery for fracture. All tissue samples were obtained after a written consent form was signed by each individual in compliance with the bioethics laws of China as well as the Declaration of Helsinki. The research was also approved by the Ethics Committee of the first affiliated hospital of Nanchang University.

Muscle biopsy

Muscle biopsies were performed from the left or right biceps brachii of patients before riboflavin therapy. The tissue was frozen and then consecutively cut into 8-um sections. These sections were stained according to standard histological and enzyme histochemical procedures with HE, modified Gomori trichrome (MGT), periodic acidic Schiff (PAS), ORO, adenosine triphosphatase (ATPase), nicotinamide adenine dinucleotidetetrazolium reductase (NADH-TR), succinate dehydrogenease (SDH), cytochrome *c* oxidase (COX) and nonspecific esterase (NSE). For electron microscopy, the specimens were initially fixed in 2.5% glutaraldehyde, subsequently in 1% osmium tetroxide, and then embedded in Epon 812. Ultrathin sections were examined through an electron microscope (JEOL-1230, Nihon Denshi, Tokyo, Japan).

Mutation screening

Mutation screening was performed in 13 affected patients and their parents. Genomic DNA (gDNA) was extracted from blood samples. Coding exons of the *ETFA, ETFB* and *ETFDH* gene were amplified using PCR with intronic primers. Primers (Supplementary Table S1) were designed on the basis of the sequences published in the Ensembl Genome Browser (*ETFA*, ENSG00000140374; *ETFB*, ENSG00000105379; *ETFDH*, ENSG00000171503). After purification, PCR products were directly sequenced with an ABI 3730 DNA Analyzer (Applied Biosystems Inc., Foster City, CA, USA). To exclude PCR errors, all nucleotide variations were sequenced in reverse. To exclude the possibility that the *ETFDH* mutation represents polymorphisms, identical genomic fragments from 100 healthy controls of Chinese origin were examined for novel mutations.

To investigate whether exon deletion/duplication of the *ETFDH* gene exists in patients with RR-MADD, a multiplex PCR was designed according to complementary DNA (cDNA) sequences of the *ETFDH* gene. The PCR reactions were carried out in a final volume of 50 µl under standard reaction conditions using Taq DNA polymerase (Takara, Dalian, China). The annealing temperature was 55 °C. The multiplex PCR sets were divided into two groups: group 1 included exons 3, 6, 7, 8, 11/12, 13 and exon 1 of *GAPDH* as the reference gene; group 2 included exons 1, 2, 4/5, 9, 10 and exon 7 of *GAPDH* as the reference gene. PCR products were screened using denaturing high-performance liquid chromatography (Transgenomic Inc., Omaha, NE, USA). The primers can be found in Supplementary Information on the journal of human genetics website (Supplementary Table S2).

Express analysis of muscular ETFDH

Total RNA of 10 patients (cases 2, 4, 6, 7, 8, 9, 10, 11, 12 and 13) was successfully isolated using the Trizol reagent from the muscular tissues of patients and controls according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Reverse transcription (RT) was performed using a PrimeScript RT Reagent Kit with gDNA Eraser (Takara) in a 20 ml reaction mixture containing 1 µg of total RNA from each individual sample. To amplify the full-length *ETFDH* cDNA, the forward primer 5′-tgttgtgtccgaccgagagt-3′ was designed in exon 1, and the reverse primer 5′-gcagaaagacattctgaaatctg-3′ was designed in exon 13. The PCR reactions were carried out in a final volume of 50 µl under standard reaction conditions using Taq DNA polymerase (Takara). The process was started with a hot start of 94 °C for 5 min, and products were amplified with 35 cycles at 94 °C for 30 s, 60 °C for 45 s and 72 °C for 2 min, followed by a 10 min extension step at 72 °C. The specificity of the PCR products was assessed by 1.5% agarose gel electrophoresis. The products were sequenced using reaction primers and two other primers, namely, 5′-gttgaagtataccctggttat-3′ and 5′-ctccattcctctcaatatcca-3′.

The muscular tissues of 13 patients were dissolved in a lysis buffer. Lanes of 120 µg of each muscular protein were separated in 12% sodium dodecyl sulfate-polyacrylamide gels. Nitrocellulose membranes (Bio-Rad Laboratories, Sundbyberg, Sweden) were probed with a primary mouse monoclonal ETFDH antibody (1:1000, 3H2BG1, Abcam Inc., Cambridge, UK) and visualized with a peroxidase-linked anti-goat IgG antibody (Santa Cruz Inc., Dallas, TX, USA) using chemiluminescence reagent (Pierce, Rockford, IL, USA). β-Actin (ZSQB-BIO Inc., Beijing, China) was used as the internal control. The average density of the bands was analyzed by Quantity One 4.6.2 software (Bio-Rad Laboratories). The western blot experiments were repeated three times.

Literature review

We searched for articles in the PubMed (http://www.ncbi.nlm.nih.gov/pubmed) and WangFang Databases (http://wangfangdata.com.cn) using the following keywords: multiple Acyl-CoA dehydrogenation deficiency, lipid storage myopathy, riboflavin responsive lipid storage myopathy and electron transfer flavoprotein dehydrogenase. There were seven articles describing mainland Chinese patients with RR-MADD caused by *ETFDH* gene mutations.[4,6,10–14] We performed the clinical and genetic reanalysis of RR-MADD patients in Mainland China using the data from our study and those in the other articles.

Statistical analysis

All data were expressed as mean ± s.d. Statistical analysis was carried out using SPSS Base 16.0 software, and comparison between groups was assessed with Student' *t*-test. Statistical significance was set at $P < 0.05$.

RESULTS

Clinical characteristics in this study

The cohort of patients included 9 male and 4 female patients, and the onset age varied from 26 to 57 years (37.9 ± 9.36; Table 1). Among the 13 patients, 4 patients had trigger factors including diarrhea, pregnancy, cold and excessive physical training; however, the remaining 9 patients had no definite trigger factors. The main clinical symptoms included limb weakness in 13 patients, particularly in the proximal lower extremities, exercise intolerance in 13 patients, weakness of the neck in 6 patients, weakness of the masseter in 5 patients, myalgia in 4 patients, muscle soreness in 3 patients and hypoesthesia in 2 patients. Patients 6 and 12 suffered from recurrent vomiting after having meals rich in fat. Abdominal ultrasound revealed severe fatty liver in 4 patients; however, other extramuscular manifestations including encephalopathy, seizure, hypoglycemia, heart failure and cardiac arrhythmia were not observed. Six patients (patients 1–4, 11 and 13) had fluctuating symptoms before diagnosis. The level of serum creatine kinase was normal (0–171 IU l^{-1}) in 1 patient and elevated to 303–8298 IU l^{-1} in 12 patients. Electromyogram was performed in 10 patients, including myogenic pattern (4 patients), neurogenic pattern (1 patient) and normal pattern (5 patients).

All patients were initially treated with riboflavin (60 mg per day), L-camitine (2 g per day) and coenzyme Q10 (90 mg per day). However, 3 patients (cases 2, 7 and 11) who had not responded well to the above-mentioned treatment in the initial 2 weeks were administered riboflavin (120 mg per day), L-camitine (2 g per day), coenzyme Q10 (90 mg day) and prednisone (30 mg per day) for the next 2 weeks. Their muscle symptoms (including muscle weakness, exercise intolerance, myalgia and muscle soreness) were completely resolved within 1

Table 1 Characteristics of clinical data of 13 patients with RR-MADD in this study

			Muscle weakness									
					Upper limb		*Lower limb*					
No.	Sex/age of onset	*Trigger factor*	*Neck*	*Masseter*	*Proximal*	*Distal*	*Proximal*	*Distal*	*Myalgia*	*Other problems*	*CK (U l^{-1})*	*EMG*
1	F/48	–	–	+	5	5	3+	5	–	Hypoesthesia	8298	NP
2[a]	M/39	–	–	+	4	5–	4	5–	–	–	896	MP
3	F/35	–	–	–	5-	5	4	5	+	–	1074	NA
4	M/57	Diarrhea	–	–	5	5	4	5	+	–	6420	normal
5	M/31	–	+	+	4–	5–	4–	5–	–	Hypoesthesia	439	MP
6	M/36	–	+	–	5	5	5–	5	+	Fatty liver, vomiting	344	MP
7[a]	F/33	Pregnancy	–	–	4	5	3	5	–	–	128	NA
8	M/35	–	+	–	5	5	4	4+	–	Soreness	346	Normal
9	M/39	Exercise	+	+	5	5	3	4+	–	Soreness	382	NA
10	M/38	–	–	–	5	5	4	5	–	Soreness, Fatty liver	1205	Normal
11[a]	M/32	–	+	–	5–	5	4	5–	–	Fatty liver	561	Normal
12	M/26	Cold	+	+	4	5	3	4	+	Fatty liver, vomiting	2356	MP
13	F/56	–	–	–	5	5	4	5–	–	–	703	Normal

Abbreviations: CK, serum creatine kinase; EMG, electromyogram; F, female; M, male; MP, myogenic pattern; NP, neurogenic pattern; NA, not available.
Muscle strength was assessed according to the modified Medical Research Council scale.
[a]Cases were treated by prednisone additionally.

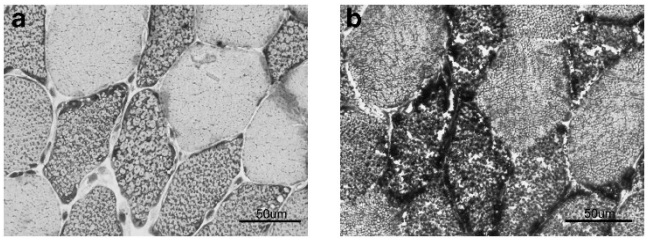

Figure 1 Myopathological characteristics in patient 3. Vacuoles in myofibers filled with lipid droplets on oil red O stain (**a**); vacuole fibers containing numerous darkly stained coarse granules on NADH stain (**b**). A full color version of this figure is available at the *Journal of Human Genetics* journal online.

month (3–28 days, 14.36 ± 5.12 days). The creatine kinase level of all patients came down to normal limits. All patients were administered riboflavin 5 mg per day for secondary prevention, but patient 3 experienced slight muscle weakness after fasting or exercise. When the dose of riboflavin was increased to 15 mg per day, the patient no longer suffered from weakness during a follow-up of 12 months.

Muscle pathological features

The hematoxylin and eosin stain showed a mild variation in fiber size with normal or slightly increased interstitial connective tissue. A few basophilic regenerating fibers were observed in five patients, but a single necrotic fiber was found only in patient 8. The characteristic features displayed that myofibers of the 13 patients were full of small round or irregular vacuoles, predominantly type I fibers (Supplementary Figure S1). The ORO stain revealed that those vacuoles were filled with lipid droplets (Figure 1a), and the vacuole fibers contained numerous coarse granules with dark staining on NADH stain (Figure 1b). On MGT, atypical ragged red fibers could be observed only in one patient. Other stains including PAS, SDH, COX, NSE and ATPase did not show any abnormalities. Ultrastructural examination revealed many lipid droplets deposited between myofibrils.

Gene mutation

All patients were confirmed to have mutations in open reading frame of the *ETFDH* gene, including a single heterozygous mutation in 5 patients (cases 1, 2, 5, 7 and 10), two heterozygous mutations in 7 patients (cases 3, 4, 6, 8, 11, 12 and 13) and three heterozygous mutations in case 9. The parents also carried the above mutations co-segregating with their offspring. The mother of case 9 had c.1395T>G, whereas the father simultaneously had c.770A>G and c.1773-1774delAT. A total of 18 different mutations were found in this study (Table 2), including 13 previously reported (c.3G>C, c.389A>T, c.524G>A, c.715G>A, c.770A>G, c.821G>A, c.1084G>A, c.1211T>C, c.1227A>C, c.1395T>G, c.1399G>A, c.l773_1774delAT and c.1828G>A) and 5 novel mutations (c.295C>T, c.303T>A, c.518T>G, c.1586A>G and c.1810G>T) (Supplementary Figure S2). The previously reported mutations mainly appeared in Chinese patients,[4,6,10,11,14] whereas c.1084G>A and c.1211T>C were identified in Japanese patients.[2,20] The five novel mutations were not found in 100 Chinese controls. All affected amino acids were conserved in most of the higher eukaryotes. In addition, copy number variation of exons in the *ETFDH* gene was not found in all patients on multiplex PCR (Supplementary Figure S3).

ETFDH expression in RR-MADD muscle

In reverse transcription PCR assays, cDNA fragments of the expected size (1974 bp) were observed in both controls and patients (Supplementary Figure S4). No additional bands were detected in the affected individuals, excluding the possibility of exon skipping due to deep intronic mutations. By sequencing, in seven patients with compound heterozygous mutations, the heterozygous mutations identified in gDNA were also found in cDNA. In three patients with single heterozygous mutations, only the transcripts with genomic mutation were detected in cases 2 and 10, indicating an absence of transcript from the no-open reading frame-mutation allele; however, case 7 had a heterozygous mutation in the cDNA-amplified products (Supplementary Figure S5).

Western blot analysis revealed a significant reduction of ETF:QO expression in 13 patients with *ETFDH* mutations. Quantitative

Table 2 Summary of the *ETFDH* gene mutation in 13 patients in this study

No.	*Exon*	*cDNA1*	*Protein1*	*Domain*	*Exon*	*cDNA2*	*Protein2*	*Domain*
Single heterozygote								
1	11	c.1395T>G	p.Y465X	UQ	?	?	?	?
2	13	c. 1773-1774delAT	p.T591TfsX2	4Fe4S	?	?	?	?
5	9	c.1084G>A	p.G362R	UQ	?	?	?	?
7	1	c.3G>C	p.M1I	MTP	?	?	?	?
10	11	c.1399G>A	p.G467R	UQ	?	?	?	?
Compound heterozygote								
3	3	c.389A>T	p.D130V	FAD	10	c.1227A>C	p.L409F	FAD
4	3	c.303T>A	p.C101X[n]	FAD	13	c.1810G>T	p.V604L[n]	4Fe4S
6	5	c.518T>G	p.I173M[n]	FAD	10	c.1211T>C	p.M404T	FAD
8	3	c.295C>T	p.R99C[n]	FAD	12	c.1586A>G	p.H529R[n]	4Fe4S
11	7	c.715G>A	p.A239T	FAD	13	c.1810G>T	p.V604L[n]	4Fe4S
12	3	c.295C>T	p.R99C[n]	FAD	7	c.821G>A	p.G274E	UQ
13	5	c.524G>A	p.R175L	FAD	13	c.1828G>A	p.G610R	4Fe4S
9	7	c.770A>G	p.Y257C	FAD	11	c.1395T>G	p.Y465X	UQ
	13	c. 1773-1774delAT	p.T591TfsX2	4Fe4S				

Abbreviations: FAD, flavin adenine dinucleotide; MTP, mitochondrial targeting peptide; n, novel mutation; UQ, ubiquinone; ?, undetected.
Patient 9 has three heterozygous mutations in the *ETFDH* gene.

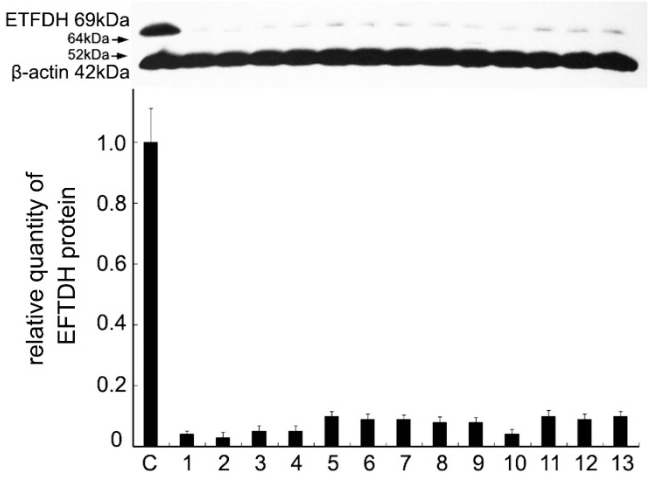

Figure 2 Western blot analysis of RR-MADD patients with the *ETFDH* mutation. Lower-molecular-weight bands were observed in patient 2 (64 kDa) and patient 9 (64 and 52k Da). Quantitative analysis showed that there was about 90% decrease in each mutant protein compared with controls. C, normal control; patient numbers are shown beneath each bar.

analysis showed that there was at least 90% decrease in each mutant protein compared with controls (Figure 2). However, the amount of expressed proteins was not statistically different among patients. In patients 2 and 9, slight lower-molecular-weight bands were observed in WB pictures because of the presence of premature terminal codons (p.Y465X and p.T591TfsX2). However, an expected 11kDa truncated protein band was not found in patient 4 owing to a premature terminal mutation (p.C101X).

RR-MADD in Mainland China

The clinical data of RR-MADD previously reported in mainland Chinese patients are summarized in Table 3. The total number of RR-MADD cases confirmed by genetic mutation had reached 148 cases (80 male and 68 female patients) since the first publication by Yan and his colleague in 2009.[4,6,10–14] The onset age varied from 2 to 64 years. The main clinical symptoms included weakness of the limbs in 146 patients (98.65%), particularly in the proximal parts, weakness of the neck in 81 patients (54.93%), weakness of the masseter in 56 patients (37.84%), myalgia in 69 patients (46.62%) and dysphagia in 31 patients (20.95%). The most frequent extramuscular manifestation was recurrent vomiting in 39 patients (26.35%). Other symptoms including fatty liver, tachypnea, palpitation, anorexia and sensory ataxia were also observed in a few patients with RR-MADD.

A total of 148 patients with RR-MADD had been identified with mutations in the *ETFDH* gene, including homozygote in 49 cases, compound heterozygote in 84 cases and single heterozygote in 15 cases. A total of 68 different causative mutations were found in 296 alleles, including 51 point mutations, 9 deletion-insertion mutations and 8 splice mutations. Mutations of the *ETFDH* gene reported in Mainland China are summarized in Figure 3 (Supplementary Table S3).[4,6,10–14] There were regional differences in the *ETFDH* mutation spectrum between northern and southern China. c.250G>A (108 alleles/296 alleles, 36.49%) was the most common mutation in southern China versus 389A>T (13 alleles/296 alleles, 4.39%) in northern China.[4,6,10,11,14] However, most patients with c.250G>A were family members and were clustered in Fujian province due to a founder effect.[10,16] c.770A>G (31 alleles/296 alleles, 10.47%) and c.1227A>C (23 alleles/296 alleles, 7.77%) were widely reported in several articles, and their prevalence was almost equivalent in southern and northern China.[4,6,10,11,14]

DISCUSSIONS

In this study, patients presented with proximal muscle weakness, exercise intolerance, muscle pain and elevated creatine kinase level. Muscle biopsy indicated lipid accumulation in myofibers. Symptoms were rapidly resolved on treatment with riboflavin. The above clinical findings led us to suspect the diagnosis of RR-MADD.[1] Finally, *ETFDH* mutations identified in these 13 cases supported the diagnosis of RR-MADD.

In our neuromuscular center serving patients for more than 14 years, we confirmed 13 RR-MADD patients out of 143 muscle biopsies between December 2011 and August 2013. RR-MADD seems to be the more common myopathy in our region compared with

Table 3 Summary of the clinical data of previously reported patients with RR-MADD

Total cases[ref]	*Sex (F/M)*	*Onset age (years)*	*Region*	*Main symptoms (number)*	*Other problems (number)*
18[4]	13/5	21.8 ± 13.5	Northern China	Proximal weakness (18), neck weakness (16), masseter weakness (14), myalgia (15)	Intermitten vomitting (8), vegetarian (11), fatty liver (1)
21[6]	8/13	24.8 ± 11.5	Northern China	Proximal weakness (21), neck weakness (17), masseter weakness (15), myalgia (11)	Intermitten vomitting (5), vegetarian (5)
53[10]	24/29	24.5 ± 12.6	Southern China	Proximal weakness (53), myalgia (29), dysphagia (12), neck weakness (9)	Intermitten vomiting (17), tachypnea (6)
23[11]	11/12	27.9 ± 9.9	Northern China	Proximal weakness (21), masseter weakness (15), neck weakness (14), myalgia (14), dysphagia (8)	Intermittent vomiting (6), sock-like hypoesthesia (2), tachypnea (4), palpitation (4)
2[12]	0/2	41, 43	Northern China	Proximal weakness (2), masseter weakness (2), neck weakness (2)	Sensory ataxia
1[13]	0/1	45	Northern China	proximal weakness, neck weakness, dysphagia	anorexia, intermittent vomiting, vegetarian
30[14]	12/18	25 ± 13.6	Southern China	Proximal weakness (30), neck weakness (23), masseter weakness (10), dysphagia (10)	Fatty liver (10), muscle wasting (7), palpitation (2), intermittent vomiting (2)

Abbreviations: F, female; M, male. A total of 34 patients were reported in Wen *et al*.,[6] but 13 patients have been described in Wen *et al*.;[4] therefore, only 21 patients are summarized in the table. The average age at onset was 25.07 ± 11.93 (reported Chinese patients) vs 37.9 ± 9.36 (our patients, $P = 0.035$).

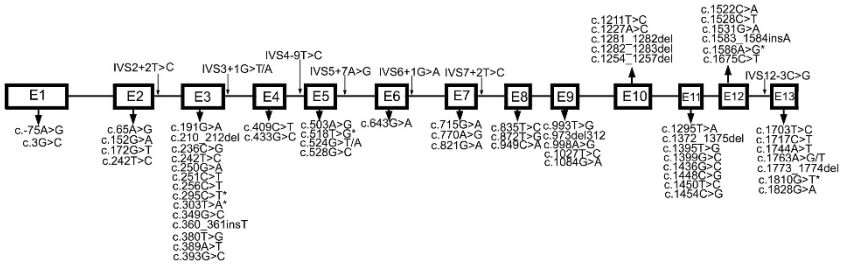

Figure 3 Schematic structure of the human *ETFDH* gene and the distribution of 74 mutations reported in a mainland Chinese patient with RR-MADD. The novel mutations identified in this study are marked with asterisks.

other Chinese regions or other countries.[1,4,21] For example, there were 18 RR-MADD cases confirmed out of about 1400 muscle biopsies in Shandong province located in northern China from 1990 to 2009,[4] and only 4 RR-MADD cases out of 9639 muscle biopsies in National Center of Neurology and Psychiatry (NCNP), the largest neuromuscular center in Japan, from 1978 to 2006.[21] However, the total number of RR-MADD cases was far greater in Mainland China than in other countries, especially in Western countries.[1,4,6,10,11,14]

The clinical features of mainland Chinese patients were to a large extent similar to those reported from other regions.[1–17] However, some clinical features of RR-MADD should be emphasized in these 13 cases. First, the age at onset was significantly higher in our center than in other mainland Chinese regions[4,6,10–14] Taiwan,[15,16] Hong Kong,[17] Japan[2,21] and Europe.[1,7,8] Therefore, our results supported the belief that RR-MADD was late-onset MADD.[1] Second, muscle symptoms were highly predominant in the 13 cases, whereas severe extramuscular features such as seizure, encephalopathy, hypoglycemia and hypotonia were common concomitant symptoms in Japanese[2,21] and European patients.[1] The muscle symptoms of 13 cases were similar to those of other cases reported from Mainland China. Third, 4 patients with fatty liver were identified among the 13 cases, similar to that found in the other series of Chinese patients (10 cases with fatty liver out of 30 cases).[14] It suggested that fatty liver might be an important extramuscular symptom in patients with RR-MADD. Finally, glucocorticoids can promote the treatment effects in some patients with RR-MADD. Glucocorticoids have long been accepted as being catabolic in nature, liberating energy substrates during times of stress to supply the increased metabolic demand of the body. However, the effects of glucocorticoids on fatty acid metabolism are less well understood.[22] In addition, glucocorticoids can alleviate the process of oxidative stress, while oxidative stress is involved in the process of pathogenesis of MADD.[23]

All patients and their parents harbored mutations of the *ETFDH* gene in this study, which coincided with the viewpoint that RR-MADD is mainly caused by *ETFDH* gene mutations.[3] Single heterozygous mutations of the *ETFDH* gene were also found in this cohort of patients similar to other reported cases,[4,14] which might be associated with mutations in the promoter region or abnormal process of protein assembly or modifications.[3] Compared with sporadic mutations in other regions, hot spot mutations were identified in the mainland Chinese population. A possible founder effect caused the high frequency of the c.250G>A mutation in Southern China, especially in Fujian province and Taiwan.[11,16] However, c.389A>T was found in many northern Chinese patients.[4,6,11] On the other hand, although the frequency of c.250G>A and c.389A>T mutations was considerably different between northern and southern China, c.770A>G and c.1227A>C were widespread hot spot mutations in the Chinese population.[4,6,10–14] Interestingly, no highly recurrent mutations were found in our 13 patients, which suggested that the mutational spectrum of RR-MADD might be broader than that expected in Mainland China.

ETF-QO mainly consists of the flavin adenine dinucleotide (FAD) and ubiquinone (UQ) binding regions, as well as a 4Fe4S cluster in the inner mitochondrial membrane.[24] Mutational data in mainland

Chinese patients showed that most mutations were mainly clustered in the FAD/UQ binding domain, which might undermine the ability of the interacting proteins to bind FAD/UQ and break down the catalytic activity of flavoproteins, as well as their folding, assembly and stability. In addition, we further demonstrated that the ETFDH protein level in the affected muscle of patients with various *EFTDH* mutations was significantly decreased, which might make the substrate less accessible to the FAD-binding site.[10,24] Therefore, the beneficial effect of riboflavin may be due to its increasing intra-mitochondrial FAD concentration, which consequently increases the stabilization of mutant flavoprotein and compensates for mitochondrial flavin and flavoprotein homeostasis.[24]

In conclusion, there have been a large number of RR-MADD cases reported in mainland Chinese patients since 2009. Our study enlarged the spectrum of clinical features and gene mutations of RR-MADD. Except for severe muscle symptoms, fatty liver and recurrent vomiting were the most common extramuscular symptoms of RR-MADD in mainland Chinese patients. The c.250G>A had a high mutation frequency in southern China, whereas c.770A>G and c.1227A>C were more geographically widespread hot spot mutations in Mainland China.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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