JNNP Online First, published on September 15, 2009 as 10.1136/jnnp.2009.176404

Riboflavin responsive lipid storage myopathy caused by *ETFDH* gene mutations

Authors rank: Bing Wen,[1] Tingjun Dai,[1] Wei Li,[1] Yuying Zhao,[1] Shuping Liu,[1] Chunhua Zhang,[2] Honghao Li,[1] Jinling Wu,[1] Danian Li,[1] Chuanzhu Yan[1,3]

[1]Laboratory of Neuromuscular Disorders and Department of Neurology, Qilu Hospital, Shandong University, Jinan, China
[2] Department of research and development, MILS International, Kanazawa, Japan
[3]The Key Laboratory for Experimental Teratology of the Ministry of Education and Institute of Medical Genetics, School of Medicine, Shandong University, Jinan, China

Correspondence to: Dr. C Yan, No.107, West Wenhua Road, Jinan, PR. of China, 250012;

czyan@sdu.edu.cn; +86-531-82169217; +86-531-86927544 (fax).

Running head: RR-LSM caused by *ETFDH* mutations

Word count (excluding title page, abstract, references, figures and tables): 3081

Keywords: *ETFDH*; Lipid storage myopathy; Multiple acyl-CoA dehydrogenase deficiency; Riboflavin

ABSTRACT

Background: Lipid storage myopathy (LSM), defined by triglyceride accumulation in muscle fibers, is a heterogeneous group of lipid metabolic disorders predominantly affecting skeletal muscle. In the past 15 years, more than 200 cases of LSM have been reported in Chinese literature, but the accurate pathogenic mechanisms are still unknown.
Objective: In order to gain more insight into the metabolic and genetic dysfunctions of LSM, we described a group of Chinese patients with LSM who were well responsive to isolated riboflavin treatment (riboflavin responsive LSM, RR-LSM).
Methods: Nineteen consecutive LSM patients collected during 1995-2007 in our Neuromuscular Laboratory who were dramatically responsive to riboflavin and presented with proximal muscle weakness, exercise intolerance and elevated serum CK but without episodic encephalopathy were subjected to pathological, biochemical and molecular analysis.
Results: On the basis of muscle pathology, all 19 patients were diagnosed as LSM. Seventeen patients were suspected to have multiple acyl-CoA dehydrogenase deficiency (MADD) according to blood acylcarnitine profiles and urine organic acid analysis. Genetic analysis identified 19 novel mutations in *ETFDH* gene in 18 patients, among which one was homozygote, sixteen were compound heterozygotes and one was single heterozygote. No pathogenic mutation was detected in *ETFA* or *ETFB* genes. Western blot analysis showed there was no significant decrease in ETF:QO expression except for one patient.
Conclusions: Our research findings suggest that the majority of Chinese patients with RR-LSM are caused by mild type of MADD with unique myopathy which is due to *ETFDH* gene mutation.

INTRODUCTION

Lipid storage myopathy (LSM) is a heterogeneous group of lipid metabolic disorders characterized by impaired oxidation of fatty acids (FAs). Several etiological factors such as metabolic enzymes deficiency interfere with lipid catabolism, leading to an accumulation of light microscopic lipid droplets in muscle fibers.

The etiopathogenisis of LSM is the impairment of FAs oxidation in muscle fibers. Four 'classic' LSMs were summarized genetically as follows (as reviewed in Bruno's article[1]): primary carnitine deficiency (PCD) due to *SLC22A5* gene mutations; Multiple acyl-CoA dehydrogenase deficiency (MADD) and myopathy with secondary CoQ10 deficiency caused by mutations in *ETFA*, *ETFB*, or *ETFDH* genes; neutral lipid storage disease (NLSD) with ichthyosis (NLSDI) or Chanarin–Dorfman syndrome (CDS) due to *CGI-58* gene mutations; and NLSD with myopathy (NLSDM) caused by mutations in *PNPLA2* gene. There were also a few case reports showing that LSM may be caused by deficiencies of long-chain L-3-hydroxyacyl-CoA dehydrogenase,[2] medium-chain acyl-CoA dehydrogenase (MCAD)[3] as well as short-chain acyl-CoA dehydrogenase (SCAD).[4, 5] Among them, only MADD and MCAD have been reported to be responsive to riboflavin.[6-11]

MADD (MIM 231680; also known as glutaric acidemia type II, GAII) as a cause of riboflavin responsive (RR)-LSM, has a wide range of severity varying from neonatal lethal form to variable milder form presenting with hypoglycemic encephalopathy or myopathy. Classical MADD is confirmed to have mutations in electron transfer flavoprotein (ETF) and ETF:ubiquinone oxidoreductase (ETF:QO).[12-14] ETF and ETF:QO are encoded by *ETFA*/*ETFB* and *ETFDH* gene, respectively. Recent work suggested that most of patients with RR-MADD have mutations in *ETFDH* gene encoding ETF:QO. In Olsen's study, the clinical manifestations of RR-MADD patients were composed of episodic encephalopathy, muscle weakness or a combination of these two symptoms. Increased lipid accumulation in muscle fibers was only found in some cases.[7]

Along with the wide use of muscle biopsy for differential diagnosis of neuromuscular disorders in China, more than 200 cases of LSM have been reported in Chinese literature in the past 15 years. Most of these patients showed muscle weakness accompanied by neither encephalopathy nor other organs involvement. They were well responsive to riboflavin or low dosage of corticosteroid. In a pilot study of a LSM child, we found a characteristic urine organic acid profile and elevated acylcarnitine level in his blood, indicating the diagnosis of MADD. Moreover, compound heterozygous mutations in *ETFDH* gene were also detected in his cultured skin fibroblast cells. These findings prompted us to gain more insight into the metabolic and genetic dysfunctions of LSM patients. Our data disclosed that the mutant *ETFDH* gene of MADD is the major cause of RR-LSM in China. These findings have important implications for the etiological diagnosis and therapeutic strategy for LSM.

PATIENTS AND MATHODS

1 Patients

We recruited patients diagnosed with RR-LSM in the Laboratory of Neuromuscular Disorders, Qilu Hospital from 1026 muscle biopsies obtained between 1995 and 2007. We began to try isolated riboflavin therapy on each patient who was pathologically diagnosed as LSM since 1995. A total of 22 LSM patients were placed on this treatment. Among them, 19 cases clinically showed a dramatic responsiveness to riboflavin. Accordingly, these 19 unrelated consecutive RR-LSM patients were included in this study. Informed consent was obtained from all the 19 patients.

The diagnosis of LSM was made based on the characteristic muscle pathological findings including small round or irregular vacuoles on hematoxylin and eosin (H&E) stain and considerably increased lipid droplets in muscle fibers on oil red O (ORO) stain.[15] While cases with lipid storage induced by steroid and obvious mitochondrial abnormalities were excluded. The diagnosis of RR-LSM was made based on clinically recovery after riboflavin treatment for about one month, including the disappearance of muscle weakness, exercise intolerance, vomiting and muscle pain.

2 Muscle pathology

Open muscle biopsy in biceps brachii was carried out in all 19 patients under local anesthesia. Muscle specimens were frozen in isopentane that was precooled in liquid nitrogen and stored at -80^{0}C. For histological examination, serial frozen sections (8μm) were stained with H&E, ORO, succinate dehydrogenase (SDH), modified Gomori trichrome (MGT), NADH-tetrazolium (NADH), cytochrome c oxidase (COX) and periodic acid Schiff (PAS).

3 Biochemical studies

Urine samples from 13 patients before riboflavin treatment and blood samples from all the 19 patients were collected for organic acid and acylcarnitine analysis, respectively. A special filter paper was wetted in urine samples and three drops of patient's blood sample were placed on another special filter paper (both filter papers were provided by MILS International). After drying in room temperature for two hours, they were sent to MILS International, Japan for analysis. Urine organic acid and blood acylcarnitine were measured by gas chromatography-mass spectrometry (GC-MS, JMS-K9 GC/MC system) and tandem mass spectrometry (MS/MS).

4 Molecular studies

Genomic DNA-based PCR amplifications and sequence analysis of *ETFA*, *ETFB*, *ETFDH* genes in 19 patients were performed. In patients with no mutation in these genes, *MFT* and *ACADM* genes sequencing was carried out. Genomic DNA and total RNA was extracted from frozen muscle biopsy specimens with a genomic DNA extract kit (Tiangen, China) and TRIzol Reagents (Invitrogen, USA), respectively. Control DNA from unaffected Chinese individuals was obtained from peripheral blood lymphocytes (samples were provided by the Institute of Medical Genetics, Shandong University). Reverse transcription was performed with a RT-PCR kit (Tiangen, China). PCR amplifications of all coding regions and intron-exon boundaries of *ETFA*, *ETFB*, *ETFDH*, *MFT*, *ACADM* genes were carried out in standard conditions, and the PCR products were subjected to direct bidirectional sequencing. All references to nucleotides or amino acids were based on the cDNA reference sequence: NM_000126.3 for *ETFA*, NM_001014763.1 and NM_001985.2 for *ETFB*, NM_004453.2 for *ETFDH*, NM_030780.2 for *MFT*, NM_001127328.1 for *ACADM* .

Western blot analysis of protein extracted from muscle homogenate was performed in 17 patients (available muscle samples were inadequate in patient 1 and 4) with a polyclonal antibody raised towards ETF:QO (Protein Tech Group, Inc, US) and a monoclonal antibody raised towards α -tubulin (Santa Cruz Biotechnology, Inc, US). The procedures for protein extraction, measurements of protein concentration and western blot analysis have been described elsewhere.[16] Three controls were normal muscle biopsies of patients who, after all tests performed, were considered free of muscle diseases. Average density of the bands was analyzed by Quantity One 4.6.2 software (Bio-Rad Laboratories, Sweden).

The initiating ATG codon is numbered as bp 1-3, and the initiating methionine is numbered as amino acid 1.

RESULTS

1 Clinical data

Patients' clinical features are summarized in table 1. Age at onset varied from 6 to 64 years, and the sex ratio was 3:1 (F/M). Proximal and trunk muscle weakness, the most common clinical features in all patients, were characterized as difficulty in keeping their head upright (usually with paraspinal muscle atrophy), lifting their arms overhead and climbing stairs. Exercise intolerance was also noticeable including extremely tiredness after walking for a short distance and fatigue of masticatory muscles during chewing. Several patients suffered from intermittent episodes of vomiting (8 out of 19) especially after meals rich in fat or protein, and the majority of the 19 patients preferred fruits and vegetables to meat and eggs (11 out of 19). Muscle tenderness or pain was frequent after exercise (15 out of 19). Almost all of the patients had fluctuating symptoms during the course of disease. Some environmental factors (such as cold, infection) can make the disease deteriorate.

No patients showed significant symptoms in brain, heart, liver or kidney except one, patient 6, who had a moderate adipose tissue infiltration in liver confirmed by puncturation biopsy. After riboflavin treatment (60 mg/d) for several months, this patient developed both significant clinical amelioration and notable improvement of liver estimated by ultrasound.

Laboratory examination revealed an elevated plasma creatine kinase (CK) level ranging from several to 20 folds of normal value in 17 patients. EMG showed diversiform changes including myogenic (5 out of 16), neurogenic (8 out of 16) and normal patterns (3 out of 16).

All of our patients regained nearly normal muscle strength after riboflavin supplementation on a dosage of 30-120 mg/d for 1-4 weeks. After about 3-6 months treatment, when they experienced a significant recovery, a discontinuation of this medicine occurred in almost all patients. Some of them had a recurrence of slight weakness after a cold or infection, and the resupplying of riboflavin was also effective.

Table 1 Summary of the clinical data of 19 patients with LSM

No	Age of onset (year)	Sex	Affected siblings	Vegetarian	Vomiting	muscle weekness						Muscle pain	CK (mmol/L)	EMG	Riboflavin (mg/d)
						Neck	Masseter	Upper limb		Lower limb					
								Prox.	Dis.	Prox.	Dis.				
1	24	F	Brother	-	-	+	+	3	5	3	5	+	677	N	30
2	23	F	-	+	+	+	+	3	4	3	4	+	513	M	40
3	13	M	-	+	+	-	-	3	4	3	4	+	218	N	60
4	12	M	-	+	+	+	+	4	5	5	4	+	2020	N	120
5	6	F	-	+	-	+	+	4	4	3	4	-	287	M	30
6	15	M	-	+	-	+	+	3	5	4	5	+	2276	N	90
7	48	F	-	+	+	+	+	4	5	4	5	+	128	N	60
8	19	M	Brother	+	-	+	-	4	5	4	5	+	247	M	30
9	17	F	-	-	-	+	-	4	5	3	4	+	513	N	100
10	22	F	-	-	-	+	+	3	5	3	5	-	478	NA	100
11	33	F	-	-	-	+	+	3	5	3	5	+	352	M	120
12	17	F	-	-	+	+	+	4	5	4	5	+	1283	Normal	150
13	16	F	-	+	+	+	+	3	5	3	5	-	611	M	90
14	15	F	-	+	-	+	+	4	5	4	5	+	5300	NA	90
15	63	F	-	+	-	-	+	4	5	5	5	-	2120	N	60
16	23	F	-	+	+	+	+	4	5	4	5	+	1998	Normal	60
17	10	F	-	-	-	+	-	4	5	3	4	+	100	NA	90
18	17	M	-	-	+	+	+	4	5	4	5	+	470	N	60

19	22	F	-	-	-	-	-	4	5	4	5	+	339	Normal	60

N, Neurogenic damage; M, Myogenic damage; NA, Not available.
CK, Serum creatine kinase (normal value: 26-178 mmol/L).
EMG, Electromyogram.
Muscle strength was assessed according to a modified Medical Research Council (MRC) muscle grading system.

2 Muscle pathology

Light microscopic assessment of histochemical stains showed a moderate variation in fiber size. Many fibers were filled with numerous vacuoles (figure 1A). There was no necrotic fiber, and only a few basophilic regenerating fibers were seen. The interstitial connective tissue was normal or slightly increased. On MGT, atypical ragged red fibers (RRFs) can be seen in three patients (figure 1B). ORO stain showed lipid droplets were prominently increased in all patients, predominantly in type I fibers (figure 1C). On SDH, diffused decrease of enzyme activity was found in 16 patients (figure 1D). Other staining including NADH, COX, and PAS didn't show any abnormality. On the basis of muscular pathology, all of 19 patients were diagnosed as LSM.

3 Biochemical studies

Organic acid analysis of 13 urine samples collected before riboflavin treatment showed an increase of a variety of dicarboxylic acids (glutarate, adipate, suberate and so on), ethylmalonic acid, 2-hydroxyglutaric acid, lactic acid and ketone bodies (table 2). The organic acid profile in urine highly supported the diagnosis of GA II in 12 of 13 samples.

Acylcarnitine analysis of 19 samples before (13 cases) or after (six cases) riboflavin supplementation revealed a combined elevation of short (C6), medium (C8, C10:1, C10) and long-chain (C14:1, C14) acylcarnitines in 17 patients (table 2). Patient 18 and 19 (before treatment) showed an approximately normal acylcarnitine profile except for a mild change in free carnitine. Free carnitine level was within normal range in 14 cases, elevated in three cases (about 1.5 times of the upper normal limit) and decreased in two cases (about a quarter of the lower normal limit). On the basis of these biochemical findings, seventeen patients were suspected to have MADD.

Table 2 Biochemical features for the 19 patients with LSM

No.	Organic acids in urine before treatment	Acylcarnitine profiles in blood
1	NA	C8,C10:1,C10,C12, Cit/Ser ↑
2	NA	C8,C10:1,C10,C12, Cit/Ser ↑
3	NA	C0,C6,C8,C10:1,C10,C12,Cit/Ser ↑
4	Glutarate, adipate, suberate, 2HG, 2HIC, 2HIV, 4HPL, lactate, KB	C8,C10,C12 ↑
5	NA	C6,C8,C10,C12,C14:1,C14 ↑
6	Glutarate, adipate,2HG, 4HPL, 2HIV, 2HIC, 2M3HB, 2HB, lactate, KB	C8,C10,C12 ↑
7	NA	C0↓C3/C2,C8,C10, Cit/Ser ↑
8	NA	C3/C2,C8,C10,C12,C14:1,C14 ↑
9	Glutarate, adipate, EMA, 2HG, KB	C10,C12,C14:1,C14 ↑
10	Adipate, suberate,2HG, lactate, KB Glutarate, EMA, 3Hsebacate, 2HIC, 3HP, 4HPL	OHC4,OHC4/C2S,C10,C12,C14:1,C14 ↑
11	Glutarate, adipate,2HG, lactic acid, KB, EMA, 4HPL	C0↓OHC4/C2S,C12,C14:1,C14 ↑
12	Glutarate, adipate, suberate,2HG, KB	OHC4/C2S,C10,C12,C14:1,C14 ↑
13	Glutarate, adipate, EMA, 2HG, KB	OHC4/C2S,C10,C12,C14:1,C14 ↑
14	Glutarate, 2HG	C3/C2,C8,C10,C12,C14:1,C14 ↑
15	Glutarate, adipate, suberate, EMA, 2HG, 4HPL, uracil	C0,C5,C6,C8,C10:1,C10,C12,C14:1,C14 ↑
16	Glutarate, adipate, EMA, 2HG, lactate, KB	C8,C10,C12,C14:1,C14 ↑
17	Glutarata, adipate, suberate, 2HG, 3Hsebacate, KB	C8,C10,C12,C10:1,C14:1,C14 ↑
18	3HG, 4HPL, lactate, glycerol	C0 ↓

19	Normal	C0 ↑

NA, not available; 2HIV, 2-hydroxyisovaleric; 2M3HB, 2-methyl-3-hydroxybutyrate; 2HIC, 2-hydroxyhexanoic acid
KB, ketone bodies; 3HG, 3-hydroxyglutaric acid; 2HG, 2-hydroxyglutaric acid: 4HPL, 4-hydroxyphenyllactic acid
EMA, ethylmalonic; 3HP, 3-hydroxypropanoic acid; 2HB, 2-hydroxybutyrate; 3HB, 3-hydroxybutyrate

4 Molecular studies

4.1 Sequencing of *ETFDH* gene

Eighteen out of 19 RR-LSM patients were confirmed to have mutations in *ETFDH* gene including one with homozygous mutation, 16 with compound heterozygous mutations and one with single heterozygous mutation. Altogether, 20 different disease-causing mutations were identified in this study (table 3) and all of them were novel except c.1084G>A (p.G362R), which was reported in Japanese.[17] There are two frequent mutations, c.1227A>C (p.L409F) mutation found in seven different chromosomes and c.770A>G (p.Y257C) mutation found in six different chromosomes. None of the 15 missense mutations as well as three splicing mutations was identified in a screen of 200 control chromosomes in Chinese individuals. All affected amino acids conserved in most of higher eukaryotes (supplemental data, figure 1).

In patient 7, 16, and 19, we identified only one heterozygous mutation in genomic DNA; however, by RT-PCR, only the transcript with this mutation was detected, indicating an absence of transcript from the other allele. It may be due to mutations in the promoter region or the nonsense mediated decay pathway resulted from mutations such as exons deletion which can't be identified by genomic sequencing. For patient 15, only the IVS3+1G>A mutation was identified in genomic sequencing, which changed the splice score from 0.24 to 0 in the donor splice site of intron 3, predicted by Neural Network software;[18] unfortunately, the available muscle sample of this patient for mRNA analysis was inadequate. In addition, cDNA analysis showed the IVS4-9T>C mutation led to a truncated transcript and the IVS12-3C>G mutation resulted in an absence of its transcript. Muscle sample of patient 6 for mRNA analysis was also not available.

Table 3 Mutations identified in *ETFDH* gene

No.	cDNA 1	Protein 1	Domain 1	PSIC score	cDNA 2	Protein 2	Domain 2	PSIC score
Homozygote								
13	c.770A>G	p.Y257C	FAD	1.178	c.770A>G c.993T>G	p.Y257C p.N331K	FAD UQ	1.178 2.490
Compound heterozygotes								
1*	c.1227A>C	p.L409F	FAD	1.911	c.872T>G	p.V291G	UQ	2.697
2*	c.1227A>C	p.L409F	FAD	1.911	c.872T>G	p.V291G	UQ	2.697
3*	c.1227A>C	p.L409F	FAD	1.911	c.393G>C	p.W131C	FAD	3.954
4*	c.1227A>C	p.L409F	FAD	1.911	c.1399G>C	p.G467R	UQ	2.355
5	c.242T>C	p.L81P	FAD	2.759	IVS4-9T>C	truncated		
7	c.770A>G	p.Y257C	FAD	1.178	~	~		
8	c.1227A>C	p.L409F	FAD	1.911	IVS12-3C>G	~		
9*	c.380T>G	p.L127R	FAD	2.650	c.1531G>A	p.D511N	UQ	2.322
10*	c.770A>G	p.Y257C	FAD	1.178	c.872T>G	p.V291G	UQ	2.697
11*	c.770A>G	p.Y257C	FAD	1.178	c.973del312	325del48	FAD&4Fe4S	
12	c.770A>G	p.Y257C	FAD	1.178	c.1084G>A• c.1399G>A	p.G362R p.G467R	UQ UQ	2.712 2.355
14	c.1227A>C	p.L409F	FAD	1.911	c. 389A>T	p.D130V	FAD	2.604
16*	c.1212T>C	p.M404T	FAD	3.266	~	~		
17*	c.1436G>C	p.R479T	UQ	0.913	c.1395T>G	p.Y465stop	PTC	

18*	**c.715G>A**	**p.A239T**	**FAD**	**1.112**	**c.1395T>G**	**p.Y465stop**	**PTC**
19	**c.1227A>C**	**p.L409F**	**FAD**	**1.911**	**~**	**~**	
Heterozygotes							
15	**IVS3+1G>A**						

~ represents no corresponding product can be detected.
* Sequence analyses of their parents have confirmed that each of the mutations were located in separate alleles. Samples were not available from parents of the other patients.
• This mutation was reported previously.[17]
▪ The PSIC scores was predicted by PolyPhen program,[19] the higher score represents the more severe damage to protein function.
Patient 2 and 13 have three mutations in *ETFDH* gene, confirmed by bidirectional sequencing and PCR-RFLP analysis.

4.2 Sequencing of *ETFA* and *ETFB* genes

There were no pathogenic mutation in *ETFA* and *ETFB* genes in 19 patient, except a synonymous mutation (c.924C>T) in *ETFB* gene in patient 3 and 9.

4.3 Sequencing of *MFT* and *ACADM* genes

MFT and *ACADM* genes were screened in patient 6 who didn't bear *ETFDH* mutation, and no disease causing mutation was found. Thus the disturbances of FAD transport from cytoplasm to mitochondrial and MCAD were unlikely in this patient. In addition, we identified a novel SNP in the coding region of *MFT* gene (c.899A>G, p.Y300C), and the gene frequency of G at this site was 0.036 out of 662 normal control chromosomes.

4.3 Western blot analysis

The density analysis of the bands showed there was no significant reduction in ETF:QO expression in all muscle samples examined except patient 7 who had a moderate decrease (figure 2).

DISCUSSION

Abnormalities of lipid catabolism as possible causes of human disease were first suggested in the late 1960s by Bradley *et al.* who first used the term 'lipid storage myopathy' to describe a young woman with proximal myopathy and excessive accumulation of lipid droplets in muscle fibers.[20] Diverse causes of this disease were postulated during the following half century. However, there is no etiological data from large cohort study. In China, LSM was first reported in 1990, and the number increased rapidly from then on. Until now, there are more than 200 cases according to Chinese literature. In our lab, we pathologically confirmed 52 LSM patients out of about 1400 muscle biopsies since 1990. LSM seems to be more common in China than in other regions. For example, there were only 47 LSM cases out of 9639 muscle biopsies in NCNP, the largest neuromuscular center in Japan (collected from 1978–2006, with the same criteria for diagnosis).[15, 21] We began to try single riboflavin on LSM patients since 1995. To our surprise, most of LSM patients (19 out of 22) were dramatically responsive to this regimen, and relapse was very rare. In the present study, we demonstrated that most of RR-LSM in Chinese population is mild form of MADD.

Patients in the present study manifested only as myopathy and other organs involvement, like brain, was seldom seen. It is most likely that the mutations in our patients may not change amino acids directly involved in the active center according to the crystal structures of ETF:QO;[22] however, they may cause conformational changes of the 3D structure by altering hydrophobicity, charge, overpacking or cavity creation at buried site[19], leading to a temperate function damage. It has been proved that the phenotype of *ETFDH* mutations in patients with mild form of MADD can be influenced by environmental factors.[13] The fact that most of these patients had fluctuating symptoms before treatment and metabolic stress (cold and infection) could worsen their conditions may indicate that each of our patients has at least one milder mutation which can be modulated by riboflavin content in diet and environmental factors, and these adjustable mutations can produce a few residual enzyme activities in cells preventing our patients from severe phenotypes. Moreover, ETF:QO was not deficient at protein level in almost all muscle samples examined, validating that the mutations are mild in another way. The detected protein may come from these adjustable mild mutations, though the mutant ETF:QO was supposed to be less active before riboflavin treatment according to Olsen's study.[7]

It has been demonstrated that FAD binding is important for the catalytic activity of flavoproteins as well as for their folding, assembly and stability.[10, 23, 24] A reduced availability of intramitochondial FAD may result in a less stable or inactive conformation of the affected protein and cause an accelerated breakdown. Thus, the supplementation of riboflavin, the precursor of FAD, may enhance the conformational stabilization of the mutant ETF:QO protein.[25] This seems to be the most reasonable explanation for the dramatic responsiveness of MADD to riboflavin. In the present study, each of the 18 patients with *ETFDH* mutations had at least one mutant allele in the FAD domain (except patient 17), resulting in an inability of apoenzyme to bind to its prosthetic group, FAD. Accordingly, the beneficial effect of riboflavin to our patients may be due to the stabilization of mutant flavoprotein by elevating FAD concentration, which consequently increases ETF:QO activity.

In pathology, 16 patients noted a decreased SDH activity according to the enzymohistochemical stain (figure 1D), which is consistent with some prior works.[7, 26, 27] FAD, also the prosthetic group of SDH, is important to the function of holoenzyme.[10, 23, 24] The conformation or stability of SDH may change and subsequently result in a decreased enzyme activity under the condition of riboflavin deficiency. Some further studies will be done in our next work to make this point clear.

In the clinical and biochemical aspects, patient 6 was quite similar to the other 18 LSM patients. But we failed to find any mutation in the candidate genes including *ETFA*, *ETFB*, *ETFDH*, *MFT* and *ACADM* on genomic level. One possibility is that patient 6 as well as the single heterozygote (patient 15) may have mutations such as deletions or duplications affecting multiple exons which can't be identified by genomic sequencing. An alternative explanation for patient 15 is that when combined with defect in another gene involved in fatty acid metabolism or respiratory chain, mutation in a single *ETFDH* allele (synergistic heterozygosity) can impair fatty acid oxidation and lead to myopathy.[28] Unfortunately, available muscle sample from these two patients was inadequate for RNA analysis. However, the typical clinical and histological phenotype, the acylcarnitine profiles and a marked responsiveness to riboflavin make the diagnosis of MADD highly likely in these two patients.

As is known to all, there is a combined elevation of short- (<C6), medium- (C6–C14) and long-chain (>C14) acylcarnitines in MADD. But the biochemical results of our patients only showed increased acylcarnitines no longer than C14. This may be ascribed to the compensative β-oxidation of very long- (C>20) and long-chain FAs (C14-C20) in peroxisomes.[29] Patients will show increased long-chain acylcarnitines in serum only when this compensation collapses. This phenomenon also suggested that our patients had a mild phenotype.

In MADD, free carnitine in serum is assumed to decrease because of the increased acylcarnitines. The acyl groups were persistently conjugated to carnitine but the deacylation of acylcarnitines was blocked, resulting in a depletion of free carnitine.[3] But most of our patients had a normal range of free carnitine in serum, suggesting a mild metabolic disturbance in them. In addition, ketonuria, which is not consistent with MADD, was found in 8 cases.[30] The exact meaning of this paradox remains to be clarified.

We conclude that most of LSM in Chinese population is mild form of MADD caused by *ETFDH* mutations involving FAD domain at least in one allele. Although this mild type of MADD with unique myopathy is dramatically responsive to riboflavin supplementation, its true mechanism remains to be clarified.

Acknowlegements: The authors thank the Institute of Medical Genetics, Shandong University for contributing the DNA of normal controls. The authors are grateful to the two anonymous reviewers for their thoughtful comments and suggestions that greatly improved the quality of this manuscript.

Funding: This study was supported by a grant from Natural Science Foundation of Shandong Province, China (the grant number is Y2002C34).

Competing interests: None.

Ethics approval: The study was approved by the medical Ethics Committee of Qilu Hospital.

Fig. 1 Pathological findings

Muscle biopsies of all patients show moderate to severe vacuolar myopathy (H&E stain: A); Atypical ragged red fibers can be seen (MGT stain: B); ORO (C) stain shows lipid accumulation mainly in type 1 fibers. As compared in the same slide, there is significant difference in SDH activity between patient (left) and normal control (right) who, after all tests performed, were considered free of muscle diseases (D). COX activity is normal (E).

Fig. 2 Western blotting and quantitative analysis of ETF:QO

Twenty-five micrograms of protein were loaded per lane. ETF:QO, ETF:ubiquinone oxidoreductase; C, a normal control; Patient numbers are shown at the top of the gel (A) and at the bottom of the figure (B).

REFERENCES

1. **Bruno C**, Dimauro S. Lipid storage myopathies. *Curr Opin Neurol* 2008; **21**: 601-6.
2. **Fryburg JS**, Pelegano JP, Bennett MJ, *et al.* Long-chain 3-hydroxyacyl-coenzyme A dehydrogenase (L-CHAD) deficiency in a patient with the Bannayan-Riley-Ruvalcaba syndrome. *Am J Med Genet* 1994; **52**: 97-102.
3. **Di Donato S**, Taroni F. Lipid strorage myopathy. In:Engel AG and Franzini-Armstrong C, eds. *Myology*. New York: McGraw-Hill 2003: 1587-621.
4. **Tein I**, Elpeleg O, Ben-Zeev B, *et al.* Short-chain acyl-CoA dehydrogenase gene mutation (c.319C > T) presents with clinical heterogeneity and is candidate founder mutation in individuals of Ashkenazi Jewish origin. *Mol Genet Metab* 2008; **93**: 179-89.
5. **Turnbull DM**, Bartlett K, Stevens DL, *et al.* Short-chain acyl-CoA dehydrogenase deficiency associated with a lipid-storage myopathy and secondary carnitine deficiency. *N Engl J Med* 1984; **311**: 1232-6.
6. **Duran M**, Cleutjens CB, Ketting D, *et al.* Diagnosis of medium-chain acyl-CoA dehydrogenase deficiency in lymphocytes and liver by a gas chromatographic method: the effect of oral riboflavin supplementation. *Pediatr Res* 1992; **31**: 39-42.
7. **Olsen RKJ**, Olpin SE, Andresen BS, *et al.* ETFDH mutations as a major cause of riboflavin-responsive multiple acyl-CoA dehydrogenation deficiency. *Brain* 2007; **130**: 2045-54.
8. **Chiong MA**, Sim KG, Carpenter K, *et al.* Transient multiple acyl-CoA dehydrogenation deficiency in a newborn female caused by maternal riboflavin deficiency. *Mol Genet Metab* 2007; **92**: 109-14.
9. **Sperl W**, Geiger R, Lehnert W, *et al.* Stridor as the major presenting symptom in riboflavin-responsive multiple acyl-CoA dehydrogenation deficiency. *Eur J Pediatr* 1997; **156**: 800-2.
10. **Saijo T**, Tanaka K. Isoalloxazine ring of FAD is required for the formation of the core in the Hsp60-assisted folding of medium chain acyl-CoA dehydrogenase subunit into the assembly competent conformation in mitochondria. *J Biol Chem* 1995; **270**: 1899-907.
11. **Didonato S**, Gellera C, Peluchetti D, *et al.* Normalization of Short-Chain Acylcoenzyme-a Dehydrogenase after Riboflavin Treatment in a Girl with Multiple Acylcoenzyme-a Dehydrogenase Deficient Myopathy. *Ann Neurol* 1989; **25**: 479-84.
12. **Goodman SI**, Binard RJ, Woontner MR, *et al.* Glutaric acidemia type II: gene structure and mutations of the electron transfer flavoprotein:ubiquinone oxidoreductase (ETF:QO) gene. *Mol Genet Metab* 2002; **77**: 86-90.
13. **Olsen RKJ**, Andresen BS, Christensen E, *et al.* Clear relationship between ETF/ETFDH genotype and phenotype in patients with multiple Acyl-CoA dehydrogenation deficiency. *Hum Mutat* 2003; **22**: 12-23.
14. **Schiff M**, Froissart R, Olsen RK, *et al.* Electron transfer flavoprotein deficiency: functional and molecular aspects. *Mol Genet Metab* 2006; **88**: 153-8.
15. **Ohkuma A**, Noguchi S, Sugie H, *et al.* Clinical and genetic analysis of lipid storage myopathies. *Muscle Nerve* 2009; **39**: 333-42.

16. **Nishino I**, Fu J, Tanji K, *et al.* Primary LAMP-2 deficiency causes X-linked vacuolar cardiomyopathy and myopathy (Danon disease). *Nature* 2000; **406**: 906-10.
17. **Yotsumoto Y**, Hasegawa Y, Fukuda S, *et al.* Clinical and molecular investigations of Japanese cases of glutaric acidemia type 2. *Mol Genet Metab* 2008; **94**: 61-7.
18. Neural Network[online]. Available at: http://www.fruitfly.org/seq_tools/splice.html Accessed October 22, 2008.
19. PolyPhen program[online]. Available at: http://coot.embl.de/PolyPhen/ Accessed October 12, 2008.
20. **Bradley WG**, Hudgson P, Gardner-Medwin D, *et al.* Myopathy associated with abnormal lipid metabolism in skeletal muscle. *Lancet* 1969; **1**: 495-8.
21. **Ohkuma A**, Nonaka I, Malicdan MC, *et al.* Distal lipid storage myopathy due to PNPLA2 mutation. *Neuromuscul Disord* 2008; **18**: 671-4.
22. **Zhang J**, Frerman FEKim JJ. Structure of electron transfer flavoprotein-ubiquinone oxidoreductase and electron transfer to the mitochondrial ubiquinone pool. *Proc Natl Acad Sci U S A* 2006; **103**: 16212-7.
23. **Nagao M**, Tanaka K. FAD-dependent regulation of transcription, translation, post-translational processing, and post-processing stability of various mitochondrial acyl-CoA dehydrogenases and of electron transfer flavoprotein and the site of holoenzyme formation. *J Biol Chem* 1992; **267**: 17925-32.
24. **Muralidhara BK**, Rathinakumar RWittung-Stafshede P. Folding of Desulfovibrio desulfuricans flavodoxin is accelerated by cofactor fly-casting. *Arch Biochem Biophys* 2006; **451**: 51-8.
25. **Henriques BJ**, Rodrigues JV, Olsen RK, *et al.* Role of flavinylation in a mild variant of multiple acyl-CoA dehydrogenation deficiency: A molecular rationale for the effects of riboflavin supplementation. *J Biol Chem* 2008.
26. **Vergani L**, Barile M, Angelini C, *et al.* Riboflavin therapy - Biochemical heterogeneity in two adult lipid storage myopathies. *Brain* 1999; **122**: 2401-11.
27. **Gempel K**, Topaloglu H, Talim B, *et al.* The myopathic form of coenzyme Q10 deficiency is caused by mutations in the electron-transferring-flavoprotein dehydrogenase (ETFDH) gene. *Brain* 2007; **130**: 2037-44.
28. **Uppin MS**, Sundaram C, Meena AK, *et al.* Lipid storage myopathies with unusual clinical manifestations. *Neurol India* 2008; **56**: 391-3.
29. **van den Bosch H**, Schutgens RB, Wanders RJ, *et al.* Biochemistry of peroxisomes. *Annu Rev Biochem* 1992; **61**: 157-97.
30. **al-Essa MA**, Rashed MS, Bakheet SM, *et al.* Glutaric aciduria type II: observations in seven patients with neonatal- and late-onset disease. *J Perinatol* 2000; **20**: 120-8.

Figure 1 Sequence alignment of human ETF:QO

Sequence alignment of human ETF:QO (UniProt accession No. Q16134) with the other species including those from Bos (Q2KIG0), Canis (A9M9P8), Mus (Q921G7), Xenopus (Q6GL88) Drosophila (Q7JWF1) and Saccharomyces (Q08822). Multiple sequence alignments were performed with clustalW. Affected residues are marked in red.

Homo

-------MLVPLAKLSCLAYQCFHALKIKKNYLPLCATRWSSTSTVPRITTHYTIYPRDK 53

Bos

-------MQVLLARLACPVYQCFHAIKIKKNYLPLCATRWSSTSVVPRITTHYTVYPRDQ 53

Canis --

Mus

-------MLVRLTKLSCPAYHWFHALKIKK-CLPLCAPRCSSTSAVPQITTHYTVHPREK 52

Xenopus

--------MLPLCKHTLHAHHCFAYFTALKNCLPSSCLKRWASSSVPRITTHYTICPRDQ 52

Drosophila

PRAFEELFPDWKEKGAPLMSALLKINRVQRLFTPALVRSVSEAAKYPRITTHYTLNPREK 180

Saccharomyces -------------MIKFTNENLIRGIRMTISAKSRHLALGTDMTRKFSLSCRFLNKANLT 47

L81

Homo

DKRWEGVNMERFAEEADVVIVGAGPAGLSAAVRLKQLAVAHEKD-IRVCLVEKAAQIGAH 112

Bos

DKRWEGVNMERFAEEADVVIVGAGPAGLSAAARLKQLAAQHEKD-IRVCLVEKAAQIGAH 112

Canis --MQDGLQPLLYLELLPTILFIPG------------IRTSDGK----------------- 29

Mus

DKRWEGVNMERFAEEADVVIVGAGPAGLSAAIRLKQLAAEQGKD-IRVCLVEKAAQIGAH 111

Xenopus

DKRWEGMNMERFAEEADVVIVGGGPAGLSAAIRLKQLAAECDKE-LRVCLVEKGAQIGAH 111

Drosophila

DGRWKEVDMERCIEEVDIVIVGGGPAGMSAAIRAKQLAAEKDQE-IRVCVVEKAAEVGGH 239

Saccharomyces

EEEKELLNEPRARDYVDVCIVGGGPAGLATAIKLKQLDNSSGTGQLRVVVLEKSSVLGGQ 107

D130V

L127 W131

Homo

TLSGACLDPGAFKELFPDWKEKG-----APLNTPVTEDRFGILTEKYRIPVPILPGLPMN 167

Bos

TLSGACLDPRALQELFPDWKEKG-----APLNTPVTEDRFGILTEKYRIPVPILPGLPMN 167

Canis ----------------------------APLNTPVTEDRFGILTEKYRIPVPILPGLPMN 61

Mus

TLSGACLDPAAFKELFPDWKEKG-----APLNTPVTEDRFAILTEKHRIPVPILPGLPMN 166

Xenopus

TLSGACLEPRALEELFPDWKERG-----APLNTPVTEDKFGILTKKHRIPVPILPGLPMN 166

Drosophila

ILSGAVVDPISLNELIPDWQEQG-----APLNTPVSKDTFSFLTSSGRISIPIFKGWPMD 294

Saccharomyces

TVSGAILEPGVWKELFPDEKSDIGIPLPKELATLVTKEHLKFLKGKWAISVPE-PSQMIN 166

Homo

NHGNYIVRLGHLVSWMGEQAEALGVEVYPGYAAAEVLFHDDGSVKGIATNDVGIQKDGAP 227

Bos

NHGNYIVRLGHLVSWMGEQAEALGVEVYPGYAAAEVLFHEDGSVKGIATNDVGIQKDGAP 227

Canis

NHGNYIVRLGHLVSWMGEQAEALGVEIYPGYAAAEVLFHEDSSVKGIATNDVGIQKDGAP 121

Mus

NHGNYIVRLGHLVSWMGEQAEALGVEVYPGYAAAEVLYHEDGSVKGIATNDVGIQKDGAP 226

Xenopus

NHGNYIVRLGHFVSWLGEQAEALGVEIYPGYAASEVLFHEDGSVKGIATNDVGIHKDGSP 226

Drosophila

NHGNYVVRLGHLVKWLGDQAEAMGVEIYPGCAASEVLFHEDGSVKGIATNDVGIAKTGAP 354

Saccharomyces

KGRNYIVSLNQVVGYLGEKAEEVGVEVYPGIAVSDLIYDENNAVKGVITKDAGISKSGKP 226

A239 Y257

Homo

KATFERGLELHAKVTIFAEGCHGHLAKQLYKKFDLRANCEPQTYGIGLKELWVIDEKNWK 287

Bos

KTTFERGLELHAKVTIFAEGCHGHLAKQLYRKFDLRANCEPQTYGIGLKELWVIDEKKWK 287

Canis

KTTFERGLELHAKVTVFAEGCHGHLAKQLYKKFDLRANCDPQTYGIGLKELWIIDEKKWK 181

Mus

KTTFERGLELHAKVTVFAEGCHGHLAKQLYKKFDLRASCDAQTYGIGLKELWIIDEKKWK 286

Xenopus

KSTFERGLELHAKVTIFGEGCHGHLAKQLYNKFNLRQNCEPQTYAIGLKELWVIDEKKWK 286

Drosophila

KDTFARGMELHAKTTIFAEGCRGHLTKQVMQKFNLNEGSEPQAYGIGLKEVWEIAPEKHQ 414

Saccharomyces

KETFERGMEFWARQTVLAEGCHGSLTKQALAKYDLRKGRQHQTYGLGIKEVWEVKPENFN 286

V291 N331

Homo

PGRVDHTVGWPLDRHTYGGSFLYHLNEGEPLVALGLVVGLDYQNPYLSPFREFQRWKHHP 347

Bos

PGRVDHTVGWPLDRHTYGGSFLYHLNEGEPLVALGFVVGLDYQNPYLSPFREFQRWKHHP 347

Canis

PGRVDHTVGWPLDRHTYGGSFLYHLNEGEPLVALGFVVGLDYQNPYLSPFREFQRWKHHP 241

Mus

PGRVDHTVGWPLDRHTYGGSFLYHLNEGEPLVAVGFVVGLDYQNPYLSPFREFQRCKHHP 346

Xenopus

PGCVEHTVGWPLDRHTYGGSFLYHLNEGEPLVALGLVVGLDYQNPFINPFRELQRWKHHP 346

Drosophila

PGLVEHTIGWPLDRFTYGGSFLYHLNEPTPTIAVGFVVGLNYKNPWLSPFQEFQRFKTHA 474

Saccharomyces

KGFAAHTMGYPLTNDVYGGGFQYHFGDG--LVTVGLVVGLDYKNPYVSPYKEFQKMKHHP 344

G362 M404

Homo

SIRPTLEGGKRIAYGARALNEGGFQSIP-KLTFPGGLLIGCSPGFMNVPKIKGTHTAMKS 406

Bos

SIQPTLEGGKRIAYGARALNEGGLQCIP-KLTFPGGLLIGCSPGFMNVPKIKGTHTAMKS 406

Canis

SIQPTLEGGKRIAYGARALNEGGFQSIP-KLTFPGGLLIGCSPGFMNVPKIKGTHTAMKS 300

Mus

SIQPTLEGGKRIAYGARALNEGGLQSIP-KLTFPGGLLIGCSPGFMNVPKIKGTHTAMKS 405

Xenopus

SVVPTLEGGNRIAYGARALNEGGFQSLP-KLTFPGGILVGCSPGFMNVPKIKGTHTAMKS 405

Drosophila

KVRDIFDGATRIAYGARAINEGGYQSIPQKLSFPGGCLVGCSAGFLNVPRIKGSHYAMKS 534

Saccharomyces

YYSKVLEGGKCIAYAARALNEGGLQSVP-KLNFPGGVLVGASAGFMNVPKIKGTHTAMKS 403

L409 V432

Homo GILAAESIFNQLTS-------------ENLQSKTIGLHVTEYEDNLKNSWVWKELYSVRN

453

Bos GILAAESIFNQLTN-------------ENLQSKTIGLDVTEYEDNLKKSWVWKELYAVRN

453

Canis GILAAESIFKQLTS-------------ENLQSKTIGLHVTEYEDNLKKSWVWKELYTVRN

347

Mus GSLAAESIFKQLTS-------------ENLQSKTTGLHVTEYEDNLKQSWVWKELHAVRN

452

Xenopus GMLAAENIFKKLTD-------------ESIQSKTQGLDVPEYEESLKKSWIWKELYSVRN

452

Drosophila GMLAAESALEAIN--------------ADSQS-TAGVEPTSYADKIKDSFVWKDLYKVRN 579

Saccharomyces

GLLAAESIFESIKGLPVLEEVEDEDAKMAMFDKEATINLESYESAFKESSIYKELYEVRN 463

G467 R479 P510 D511

Homo

IRPSCHGVLGVYGGMIYTGIFYWILRGMEPWTLKHKGS-DFERLKPAKDCTPIEYPKPDG 512

Bos

IRPSCHSILGVYGGMIYTGIFYWIFRGMEPWTLKHKGS-DSDKLKPAKDCTPIEYPKPDG 512

Canis

IRPSCHGILGVYGGMIYTGIFYWIFRGMEPWTLKHKGS-DSDQLKPAKDCTPIEYPKPDG 406

Mus

IRPSCHGILGVYGGMIYTGIFYWILRGMEPWTLKHKGS-DSDQLKPAKDCTPIEYPKPDG 511

Xenopus

IRPSCHGPLGLYGGMVYTGIFYWMLRGKEPWTLKHKGL-DSNQLKPAKDCVPIEYPKPDG 511

Drosophila

VHPSFHNPLGLYGGLVLSG-FSIFMGGREPWTLKHGPQ-DHESLQLASASEPIVYPKPDG 637

Saccharomyces

IRPSFSGKLGGYGGMIYSGIDSLILKGKVPWTLKFDEKNDGEILEPASKYKPIEYPKPDG 523

Homo

QISFDLLSSVALSGTNHEHDQPAHLTLRDDSIP--VNRNLSIYDGPEQRFCPAGVYEFVP 570

Bos

QISFDLLSSVALSGTNHEHDQPAHLTLKDDSVP--VNRNLSIYDGPEQRFCPAGVYEFVP 570

Canis

QISFDLLSSVALSGTNHEHDQPAHLTLRDDSIP--VNRNLSLFDGPEQRFCPAGVYEFVP 464

Mus

QISFDLLSSVALSGTNHEHDQPAHLTLKDDSIP--VNRNLSIYDGPEQRFCPAGVYEFVP 569

Xenopus

KLSFDLLSSVALSGTNHDHDQPAHLTLKDDSLP--VNKNLAIYDGPEQRFCPAGVYEFVP 569

Drosophila

KVSFDLLSSVALTGTNHEGDQPAHLTLKDDRIP--VDHNLALYEGPEQRFCPAGVYEYVP 695

Saccharomyces

VISFDILTSVSRTGTYHDDDEPCHLRVPGQDMVKYAERSFPVWKGVESRFCPAGVYEFVK 583

Homo VEQG-DGFRLQINAQNCVHCKTCDIKDPSQNINWVVPEGGGGPAYNGM 617

Bos VEQG-DGFRLQINAQNCVHCKTCDIKDPSQNINWVVPEGGGGPAYNGM 617

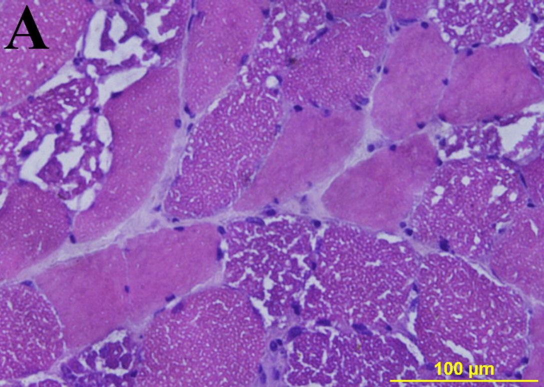
Canis LEQG-DGFRLQINAQNCVHCKTCDIKDPSQNINWVVPEGGGGPAYNGM 511

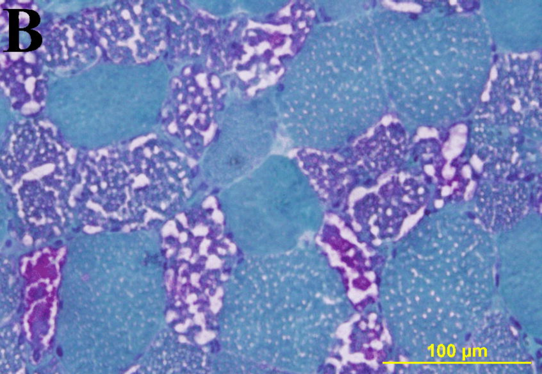
Mus LEQG-DGFRLQINAQNCVHCKTCDIKDPSQNINWVVPEGGGGPAYNGM 616

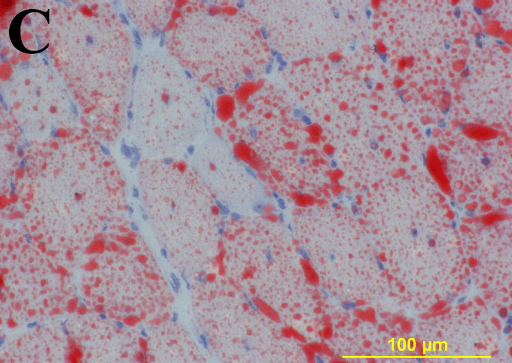
Xenopus LESG-EGSRLQINAQNCVHCKTCDIKDPSQNINWVVPEGGGGPAYNGM 616

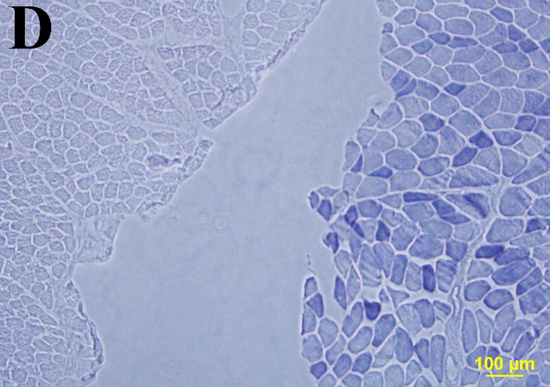
Drosophila NEEG-GNMKLQINAQNCIHCKTCDIKDPKQNINWVVPEGGGGPAYNGM 742

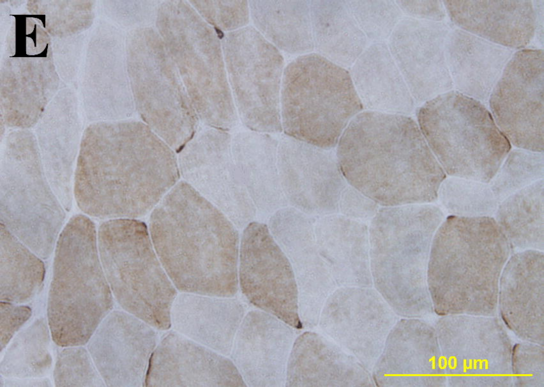
Saccharomyces DEKSPVGTRLQINSQNCIHCKTCDIKAPRQDITWKVPEGGDGPKYTLT 631

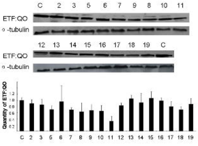

A
100 μm


B
100 µm


C
100 µm


D
100 µm


E
100 µm


A
C 2 3 5 6 7 9 8 10 11
ETF:QO
α-tubulin
12 13 14 15 16 17 18 19 C
ETF:QO
α-tubulin
B
Quantity of ETF:QO
1.2
1.0
0.8
0.6
0.4
0.2
C 2 3 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

JNNP

Riboflavin responsive lipid storage myopathy caused by ETFDH gene mutations

Bing Wen, Tingjun Dai, Wei Li, Yuying Zhao, Shuping Liu, Chunhua Zhang, Honghao Li, Jinling Wu, Danian Li and Chuanzhu Yan

J Neurol Neurosurg Psychiatry published online September 15, 2009