

# Clinical features and mutations in seven Chinese patients with very long chain acyl-CoA dehydrogenase deficiency

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**Background:** Very long chain acyl-CoA dehydrogenase deficiency (VLCADD) is an inherited metabolic disease caused by deleterious mutations in the *ACADVL* gene that encodes very long chain acyl-CoA dehydrogenase (VLCAD), and which can present as cardiomyopathy in neonates, as hypoketotic hypoglycemia in infancy, and as myopathy in late-onset patients. Although many *ACADVL* mutations have been described, no prevalent mutations in the *ACADVL* gene have been associated with VLCADD. Herein, we report the clinical course of the disease and explore the genetic mutation spectrum in seven Chinese patients with VLCADD.

**Methods:** Seven Chinese patients, from newborn to 17 years old, were included in this study. Tandem mass spectrometry was performed to screen for VLCAD deficiency. All exons and flanking introns of the *ACADVL* gene were analyzed using polymerase chain reaction and direct sequencing. Online analysis tools were used to predict the impact of novel mutations.

**Results:** All cases had elevated serum levels of tetradecanoylcarnitine (C14:1) which is the characteristic biomarker for VLCADD. The phenotype of VLCADD is heterogeneous. Two patients were hospitalized for hypoactivity and hypoglycemia shortly after birth. Three patients showed hepatomegaly and hypoglycemia in infancy. The other two adolescent patients showed initial manifestations of exercise intolerance or rhabdomyolysis. Three of the patients died at the age of 6-8 months. Eleven

different mutations in the *ACADVL* gene in the 7 patients were identified, including seven reported mutations (p.S22X, p.W427X, p.A213T, p.G222R, p.R450H, c.296-297delCA, c.1605+1G>T) and four novel mutations (p.S72F, p.Q100X, p.M437T, p.D466Y). The p.R450H and p.D466Y (14.28%, 2/14 alleles) mutations were identified in two alleles respectively.

**Conclusions:** The clinical manifestations were heterogeneous and *ACADVL* gene mutations were heterozygous in the seven VLCADD Chinese patients. R450H may be a relatively common mutation in Asian populations. The genotype and phenotype had a certain correlation in our patients.

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**Key words:** follow-up; mutation; very long chain acyl-CoA dehydrogenase; VLCAD deficiency; treatment

## Introduction

Very long-chain acyl-CoA dehydrogenase deficiency (VLCADD, OMIM #201475) is an autosomal recessive inherited metabolic disorder of fatty acid oxidation. The prevalence is approximately 1 in 85000 according to the newborn screening program.<sup>[1]</sup> Very long-chain acyl-CoA dehydrogenase (VLCAD, EC 1.3.99.13) is a member of the family of acyl-CoA dehydrogenases that catalyzes the initial rate-limiting step of long-chain fatty acid metabolism in the spiral of mitochondrial  $\beta$ -oxidation, and along with enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase, it helps to complete the cycles of  $\beta$ -oxidation. VLCAD is a mitochondrial inner-membrane-associated protein encoded by the *ACADVL* gene (OMIM 609575). The *ACADVL* gene is about 5.4 kb long, located on chromosome 17p13.1, and containing 20 exons that encode a 655-amino-acid protein.<sup>[2,3]</sup> The *ACADVL* gene mutational spectrum is wide and more than 110

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mutations have been identified (<http://www.hgmd.cf.ac.uk/ac/index.php>).

The clinical presentation of VLCAD deficiency is heterogeneous, with phenotypes of different severities and disparate ages at the onset of the clinical manifestations. Patients with VLCADD can be clinically categorized as having one of three forms.<sup>[4-6]</sup> The neonatal onset form presents with cardiomyopathy, hepatic encephalopathy, Reye-like syndrome, and sudden infant death syndrome. The cardiomyopathy is the most common presenting symptom, which causes high morbidity and mortality in this form. The infancy onset form usually presents with recurrent hypoketotic hypoglycemia and hepatic dysfunction. However, the hepatic phenotype of infancy will often become a myopathic phenotype during childhood and adolescence. The late onset form always presents with myopathies, including muscle weakness, myalgia, and episodes of rhabdomyolysis triggered by fasting or vigorous physical exercise.

Tandem mass spectrometry (MS/MS) is used to detect blood acylcarnitine intermediates in the diagnosis of VLCADD, with tetradecanoyl-carnitine (C14:1) serving as the disease-specific marker.<sup>[7]</sup> However, to make a definite diagnosis, molecular genetic analysis or VLCAD enzyme activity assays are needed.<sup>[8-11]</sup> Herein, we present our analysis of the clinical course of the disease and genetic mutation spectrum of 7 Chinese patients with VLCADD.

## Methods

### Subjects

Seven Chinese patients with VLCAD deficiency from 7 unrelated families were recruited at the Department of Pediatric Endocrinology, Genetics and Metabolism, Xinhua Hospital, Shanghai Jiao Tong University School of Medicine from September 2006 to October 2012. The symptoms and physical findings were retrospectively collected. Informed consent was obtained from the patients and the parents.

### Acylcarnitine and urine organic acid profile by MS/MS and gas chromatography/mass spectrometry (GC/MS)

The acylcarnitine levels in dried blood spots were measured by MS/MS (AB Sciex, API 4000). The organic acid levels in urine were measured by GC/MS (Shimadzu Limited, QP2010). Sample preparation and detection procedures were based on methods reported previously.<sup>[12]</sup>

### Gene mutation analysis

DNA was extracted from white blood cells using the TIANamp Blood DNA Kit (Tiangen Biotech Co., Ltd,

China). The exonic and flanking intronic regions of the *ACADVL* gene were amplified by PCR from genomic DNA templates. Primers were designed using the primer 5.0 and a more detailed description of the design parameters is available on request. Amplified fragments were directly sequenced by a commercial sequencing service (Shanghai GeneCore Biotechnologies, China). The reference *ACADVL* gene sequence was GenBank NM\_000018.

### Analysis of novel missense mutations

The presence of identified variant alleles in 50 healthy individuals (100 control chromosomes) was used to ascertain the pathogenicity of variant alleles. Four available online analysis tools (PolyPhen-2, <http://genetics.bwh.harvard.edu/pph2/>; UniProt, <http://www.uniprot.org/>; Project HOPE; and Swiss-PDBViewer 4.0) were used to predict the effect of the novel missense mutations. To analyze the evolutionary conservation of the mutated residues, an inter-species alignment of homologous VLCAD proteins was performed using the ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). The human VLCAD protein sequence (NCBI accession number NP\_000009.1) was used as the reference sequence.

## Results

### Acylcarnitines and urine organic acid profiles

Analysis of blood acylcarnitine profiles of patients with a preliminary diagnosis of VLCADD by MS/MS indicated increased levels of long chain acylcarnitines, with C14:1 carnitine as the predominant acylcarnitine, in all patients when they were initially diagnosed (Table). Significantly elevated C14:1 carnitine, ten times higher than the upper limit of the normal range (0.01-0.3  $\mu\text{mol/L}$ ), was found in patients 1, 2, and 6. Mildly elevated C14:1 carnitine was found in patients 3, 4, and 5, along with low free carnitine levels. Urine organic acid analysis by GC/MS showed dicarboxylic aciduria in patients 1, 3, and 4.

### Clinical features at onset and clinical course

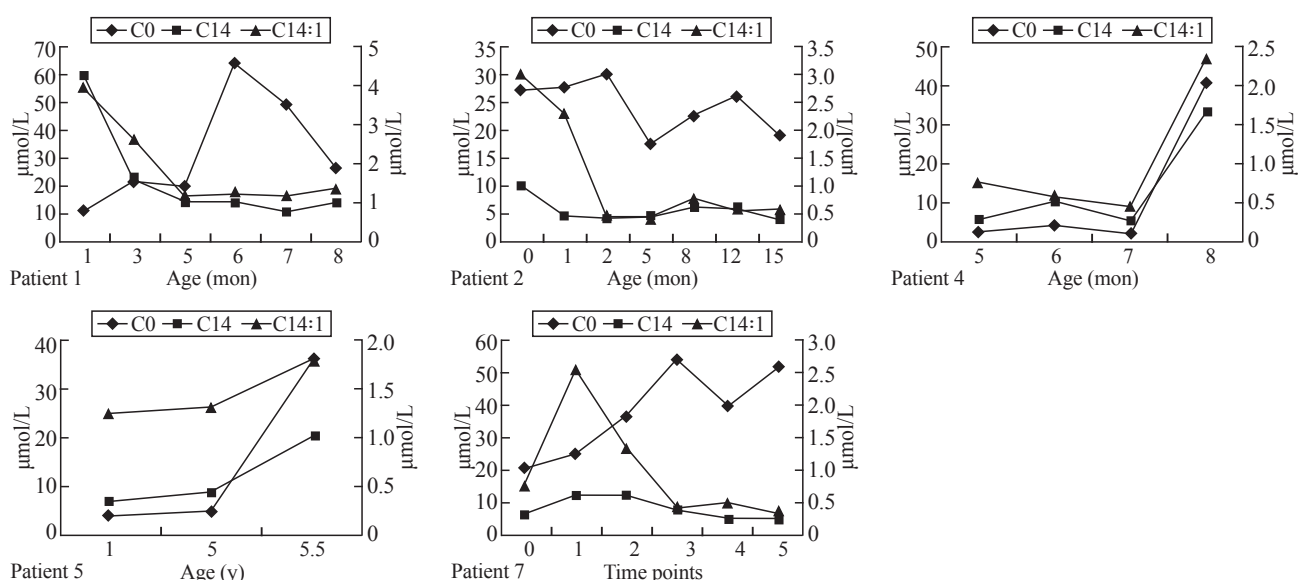
The onset age, clinical findings and laboratory investigations of the seven patients are summarized in the Table.

Patient 1 presented with hypoactivity, hyporeflexia, and hypoglycemia at 48 hours of life. He was treated with medium-chain triglycerides (MCT)-rich milk and L-carnitine, after which he experienced a subjective improvement. C14 and C14:1 carnitine decreased gradually after treatment (Fig. 1). He died from acute infection at the age of 8 months. His elder sister died from similar symptoms at 72 hours of life.

**Table.** Results of clinical characteristics and molecular analysis in seven VLCADD patients

Pt.ID	Sex	Age of onset	Clinical findings	FPG (mmol/L)	AST (U/L)	ALT (U/L)	CK (U/L)	C0 (μmol/L)	C14:1 (μmol/L)	<i>ACADVL</i> cDNA substitutions	Predicted amino acid change
1	M	At birth	Hypoglycemia, hyporeflexia, hypoactivity	0.60↓	155↑	137↑	1520↑	11.30	4.05↑	c.65C>A	p.S22X
2	M	At birth	Hypoglycemia, metabolic acidosis, neonatal pneumonia	2.70↓	50.6↑	295↑	8940↑	27.44	3.07↑	c.1280G>A	p.W427X
3	F	5 mon	Hypoglycemia, hypotonia, paleness, hepatomegaly	3.10↓	ND	36	235	2.32↓	0.61↑	c.296-297delCA	p.Q100V/Codon102X
4	M	5 mon	Hypoglycemia, hepatomegaly, developmental delay	4.28	36	91↑	224	2.87↓	0.76↑	c.298C>T	p.Q100X*
5	M	1 y	Hepatomegaly, seizure, developmental delay	5.19	12	28	180	4.53↓	1.27↑	c.1349G>A*	p.R450H*
6	M	17 y	Muscle weakness, muscle pain, rhabdomyolysis, myoglobinuria	4.60	167↑	844↑	20000↑	14.32	3.33↑	c.1396G>T*	p.D466Y*
7	F	12 y	Muscle weakness, myoglobinuria, rhabdomyolysis	4.20	456↑	3149↑	1429↑	21.18	0.80↑	c.215C>T*	p.S72F*
										c.664G>C	p.G222R
										c.1605+1G>T*	Frameshift
										c.637G>A*	p.A213T*
										c.1310T>C*	p.M437T*
										c.1396G>T*	p.D466Y*
Reference				3.9-6.0	10-42	0-75	22-269	10-60	0.01-0.3		

VLCADD: very long chain acyl-CoA dehydrogenase deficiency; Pt.ID: patient identification number; M: male; F: female; FPG: fasting plasma glucose; AST: aspartate aminotransferase; ALT: alanine aminotransferase; CK: creatine kinase; ND: not determined. \*: novel variants of the gene.



**Fig. 1.** The changes in free carnitine (C0), C14, and C14:1 carnitine levels of patients 1, 2, 4, 5, and 7 during the treatment. The left Y-axis of coordinates denotes the value of C0 and the right denotes C14 and C14:1 levels; for patient 7, the X-axis of coordinates denotes the time points at age 12 y, 12 y 1 mon, 12 y 2 mon, 12 y 5 mon, 12 y 10 mon, and 12 y 11 mon, respectively.

Patient 2 showed the symptoms of hypoglycemia and acidosis shortly after birth. He was also identified as a potential VLCADD patient through the neonatal MS/MS screening program at another hospital. He had been on special formula (Monogen, SHS) containing 90% MCT and 10% long-chain fat since the preliminary diagnosis. No obvious abnormality was observed at the last follow-up when he was 15 months old. Echocardiographic findings showed mild left ventricular hypertrophy. The levels of C14 and C14:1 carnitine decreased over time, but were slightly higher than the normal range (Fig. 1).

Patient 3 presented with hypoglycemia, hepatomegaly and hypotonia at 5 months of age. Echocardiographic findings showed pericardial effusion and cardiac insufficiency. Patient 4 exhibited symptoms at 5 months of age, which involved hepatomegaly, hypotonia, developmental delay, and repeated diarrhea. Electrocardiography analysis did not show obvious abnormalities. Although patients 3 and 4 were treated with MCT-rich formula and L-carnitine, they died from acute infection at the age of 6 and 8 months, respectively. The elder sister of patient 3 and 4 died from similar symptoms at 6 months old.

Patient 5 presented with hypoglycemia and hepatomegaly at the age of 1 year. He did not receive systematic treatment until 5 years old and showed seizure, hepatomegaly, and mental and motor retardation. Subsequently, he was treated with a low-fat diet, L-carnitine, and vitamin B2. The free carnitine in patients 4 and 5 returned to normal after treatment, while C14 and C14:1 increased (Fig. 1). C14:1 carnitine in patients 3, 4 and 5 was low on initial evaluation because of systemic carnitine deficiency (Table).

Patient 6 exhibited repeated episodes of rhabdomyolysis induced by infection or vigorous exercise at 17 years of age. His electromyogram (EMG) showed myogenic damage. The management included avoiding fasting, low-fat diet, L-carnitine and MCT oil initially; however, he could not tolerate MCT oil due to vomiting. He still encounters recurrent rhabdomyolysis 1-2 times per year induced by vigorous exercise or infections.

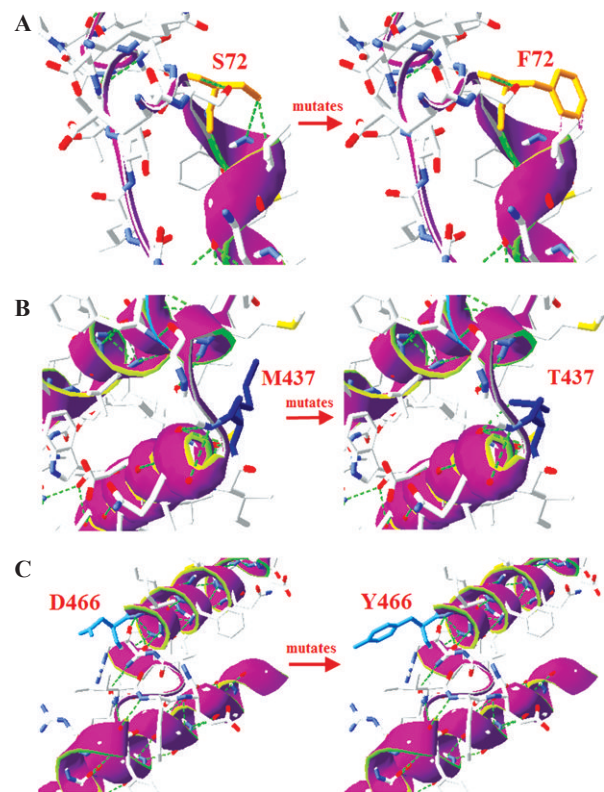
Patient 7 presented with muscle pain, muscle weakness, rhabdomyolysis, and myoglobinuria after exercise when she was 12 years old. EMG showed myogenic damage. Muscle biopsy indicated diffused accumulation of lipid droplets in the myofibers. The management of this patient included avoiding fasting, placement on a low-fat diet supplemented with L-carnitine, MCT oil, uncooked cornstarch at bedtime, and administration of fenofibrate (100 mg per day). After treatment, her muscle strength was improved obviously without recurrence of rhabdomyolysis. The levels of aspartate aminotransferase, alanine aminotransferase, creatine kinase, C14 and C14:1 carnitines were almost returned to the normal range (Fig. 1). Fenofibrate administration was the main difference in therapy utilized for patients 6 and 7, suggesting that fenofibrate was effective in reducing the recurrence of rhabdomyolysis.

### Molecular analysis

Eleven different mutations in the *ACADVL* gene including 6 missense, 4 null, and 1 splice-site mutations were identified in 7 patients with a total mutation detection rate of 92.9% (13/14), while only one heterozygous mutation was detected in patient 7 (Table). The p.R450H and p.D466Y mutations were accounted for 14.28% (2/14 alleles), respectively. The p.S72F, p.Q100X, p.M437T, and p.D466Y mutations were novel and absent in 50 unaffected individuals. The sequence alignment of homologous VLCAD proteins from various species indicated that the affected amino acids, S72, M437, and D466 are highly conserved in humans, orangutan, bovine, rat, mouse, and zebrafish.

### Prediction of functional effect on VLCAD protein

The novel missense mutations p.S72F and p.M437T



**Fig. 2.** Three-dimensional dimeric structure of very long chain acyl-CoA dehydrogenase (VLCAD): ribbon diagram (PDB 3B96) with mutation sites is shown in red. The side chain group changes of mutant amino acids of VLCAD enzyme. **A:** p.S72F (yellow), the mutant F72 residue is bigger and more hydrophobic than the wild-type S72 residue, the differences likely affect hydrogen bond formation; **B:** p.M437T (blue), the mutant T437 residue is smaller and less hydrophobic than the wild-type M437 residue, which is likely to alter hydrogen bond formation; **C:** p.D466Y (cyan), the Y466 mutant residue is bigger and more hydrophobic than the wild-type D466 residue. D466 is negatively charged, while Y466 is neutral. This mutation is likely to affect hydrogen bond formation and perturb the interactions of VLCAD with other molecules.

were predicted to be "probably damaging", and p.D466Y to be "possibly damaging" by PolyPhen-2. Project HOPE and Swiss-PdbViwer 4.0 were used to predict the changes in the secondary structure of the mutated VLCAD (Fig. 2). The mutant Phe72 residue is bigger and more hydrophobic than the corresponding Ser in the wild-type. The differences in size and hydrophobicity would affect hydrogen bond formation and disturb the interaction between subunits in VLCAD. The mutant Thr437 residue is smaller and less hydrophobic than the corresponding Met in the wild-type. The mutant Tyr466 residue is also bigger and more hydrophobic than the corresponding Asp in the wild-type. Furthermore, replacement of negatively-charged Asp466 by a neutral charged Tyr would also affect hydrogen bond formation and could alter the interaction of VLCAD with other molecules. The amino acids corresponding to the mutations p.S72F, p.M437T and p.D466Y were

all located in the catalytic region of VLCAD, which was annotated by UniProt. Meanwhile, p.M437T and p.D466Y induced amino acid changes that were also likely to perturb the interaction of VLCAD with its cofactor, flavine-adenine dinucleotide (FAD). The amino acid corresponding to the mutation p.D466Y was only four residues away from the catalytic center of the VLCAD, located at the amino acid position 462. The functions of the VLCAD protein could be directly affected by these mutations.

## Discussion

MS/MS and GC/MS have enabled the diagnosis of many inherited metabolic diseases in China during the past decade. We reported three VLCADD patients out of 3070 high-risk patients from 2002 to 2006.<sup>[13]</sup> To date, only a few groups have reported a small cohort of patients with VLCADD in Asia. Tajima et al<sup>[14]</sup> reported 34 Japanese patients with VLCADD in 2005. Two newborns with VLCADD were identified in the screening of 346 905 newborns from 1997 to 2006 in Japan.<sup>[14]</sup> Till now, there is no case of VLCADD identified by newborn screening (NBS) in more than 500 000 newborns in our NBS center (unpublished data). In this study, only patient 2 was identified as having VLCADD through newborn screening. It seems that VLCADD may be a rare disease in Asia. Molecular genetic analysis to diagnose a Chinese patient with VLCADD was firstly reported in Hong Kong in 2007.<sup>[15]</sup> In this study, we reported the clinical course including the diagnosis, treatment, follow-up and genetic mutation spectrum in the largest cohort of Chinese patients with VLCADD to date.

Increased levels of C14:1 carnitine were observed in all patients. The onset age, clinical findings and laboratory investigations of the seven patients were different. Compared with the myopathic type of patient 6 who had markedly increased C14:1, mildly elevated C14:1 carnitine and low free carnitine were found in patients 3, 4 and 5 though their clinical manifestations were more severe. It was indicated that C14:1 may not adequately reflect the severity of the disease with concomitant carnitine deficiency.

The molecular basis of VLCADD has been studied in a relatively large panel of patients.<sup>[4,16,17]</sup> No single mutation has been identified to be predominant among symptomatic patients.<sup>[4]</sup> Screening analysis of the *ACADVL* gene in our 7 patients led to the identification of eleven different mutations, indicating that the mutational spectrum of VLCADD in Chinese patients is also highly heterogeneous. The p.R450H and p.D466Y mutations were relatively common mutations

in our patient group, accounting for 14.28% (2/14 alleles) of the total mutations. The molecular analysis of 17 Japanese VLCADD patients showed that the most prevalent mutation was p.R450H (11.76%, 4/34 alleles), suggesting that p.R450H may be a relatively common mutation in Asian population.<sup>[14,16]</sup> Four novel mutations were identified, including three missense mutations p.S72F, p.M437T, and p.D466Y, and one null mutation, p.Q100X. Alignment of homologous VLCAD revealed that S72, M437, and D466, the wild-type amino acids corresponding to the mutated sites, were highly conserved in different species. The prediction of the consequence of these novel missense mutations by polyphen-2 suggested that these were likely to alter the function of the mutant VLCAD protein. The 3D protein model showed that novel missense mutations changed the specific size, charge, and hydrophobicity of the corresponding amino acids and were likely to perturb protein function.

Several studies<sup>[4,17,18]</sup> have addressed the possible genotype-to-phenotype correlations in VLCADD. It was reported that individuals with two null mutations (such as stop-, frameshift-, and severe-splicing mutations) usually have a more severe and earlier presentation, whereas individuals with at least one missense mutation have a milder and later presentation.<sup>[4]</sup> According to the clinical features of the patients in our study, there was a certain degree of correlation between the genotype and phenotype. Patients 1 and 2 with two null mutations had onset shortly after birth and showed more severe presentations. The other 5 patients were identified with at least one detectable missense mutation and had milder and later presentations. The p.R450H mutation was found in patients 3 and 4 and defined to be a temperature-sensitive mild mutation with residual VLCAD enzyme activity.<sup>[19,20]</sup> The p.D466Y and p.S72F novel mutations, detected in patients 3, 4 and 7, were predicted to be "probably damaging" mutations by PolyPhen-2. The poor outcome of patients 3 and 4 revealed that some patients who carried missense mutations may also be at risk for metabolic decompensation during acute stress. Patient 5 was heterozygous for p.G222R and c.1605+1G>T mutations. Though the reported p.G222R and c.1605+1G>T mutations were predicted to associate with a significant decrease in residual enzyme activity,<sup>[21]</sup> the progression of patient 5 was chronic and subacute. Patient 6 was heterozygous for p.A213T and p.M437T mutations. The reported p.A213T mutation was predicted not to alter enzymatic activity and only slightly change the protein stability.<sup>[22]</sup> The p.M437T novel mutation appeared to be a mild mutation since it was correlated with a milder and later presentation in patient 6, which was consistent with the prediction of "possibly damaging" by PolyPhen-2.

Current treatments for VLCADD are based on a low-fat diet, supplemented with MCT oil, L-carnitine, and fasting precautions. The earlier diagnosis and treatment are important to improve the prognosis of VLCADD. Though patient 2 exhibited severe phenotype, his clinical course did not show obvious symptoms because he was diagnosed in a timely manner and treated early. Recent data suggested that the PPAR agonist bezafibrate could improve the fatty acid oxidation capacities in fibroblasts from the myopathy form of VLCADD, by enhancing the residual enzyme activity through stimulation of *ACADVL* gene expression.<sup>[23-25]</sup> With administration of fenofibrate, another PPAR agonist, the clinical manifestations and biochemical parameters of patient 7 were significantly improved, suggesting that PPAR agonist might be an effective treatment for myopathy patients with VLCADD.

In our study, a total of seven Chinese patients with VLCADD were diagnosed. The clinical phenotype and the biochemical analysis were coincident with the reported clinical spectrum of VLCADD symptoms. Molecular analysis of the *ACADVL* gene expression is used as the gold standard to confirm the diagnosis. In the seven patients, seven reported mutations and four novel mutations were identified. The genotype and phenotype had a certain correlation in our patients, but a deeper insight of the correlation may be obtained by functional studies of the novel mutations. The earlier diagnosis and treatment of VLCADD is critical to the improvement of the prognosis of the patients.

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**Ethical approval:** The study was approved by the Ethics Committee of Xinhua Hospital Affiliated to Shanghai Jiaotong University School of Medicine, and informed consent was obtained from the study participants.

**Competing interest:** The authors declared no conflicts of interest.

**Contributors:** Rui-Nan Zhang and Yi-Fan Li contributed equally to the study. All authors reviewed relevant articles and they contributed to the manuscript and approved the final version.

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