Characterization of 31 Patients with Riboflavin-Responsive Multiple acyl-CoA Dehydrogenase Deficiency

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Aims: To evaluate the clinical, pathological, and genetic features of patients with riboflavin-responsive multiple acyl-CoA dehydrogenase deficiency (RR-MADD).

Methods: Thirty-one patients with RR-MADD admitted to our hospital from January 2005 to November 2020 were enrolled, and their clinical data were collected. Pathological characteristics of the muscle tissue and possible pathogenic gene mutations were analyzed.

Results: The most common clinical features in all patients were symmetrical proximal muscle weakness. Laboratory examination revealed elevated levels of creatine kinase, homocysteine, and uric acid, acylcarnitines, and organic acid. The muscle biopsy revealed typical pathological changes like lipid deposition. Genetic analysis identified ETFDH mutations in 29 patients, among which one had homozygotes, 19 had compound heterozygotes, 7 had heterozygous mutations, and 2 had heterozygous mutations of both ETFDH and ETFA. Two patients had no pathogenic gene mutations. All patients were treated with riboflavin, and their symptoms improved, which was consistent with the diagnosis of RR-MADD.

Conclusion: The clinical manifestations and genetic test results of patients with RR-MADD are heterogeneous. Therefore, a comprehensive analysis of clinical, pathological, and genetic testing is essential for the early diagnosis of RR-MADD.

INTRODUCTION

Lipid storage myopathies (LSMs) are considered a heterogeneous group of genetic disorders characterized by pathological lipid deposition in muscle fibers. At present, the pathological diagnosis of LSMs accounted for approximately 3% of muscle specimens in our center, in which multiple acyl-CoA dehydrogenase deficiency (MADD) is the most common type. MADD is a rare autosomal recessive metabolic disorder of fatty acid, amino acid, and choline, which is caused by the deficiency of electron transfer flavoprotein (ETF) encoded by ETFA and ETFB or electron transfer flavoprotein ubiquinone oxidoreductase (ETF: QO) encoded by ETFDH. The severity of MADD varied from a neonatal-onset lethal form to a variable milder late-onset form presenting with myopathy. Riboflavin-responsive MADD (RR-MADD) is a special type of MADD that can be dramatically resolved by riboflavin treatment. Olsen et al. revealed that ETFDH deficiency is a major cause of RR-MADD. Early recognition of RR-MADD is crucial to improve the prognosis of patients. This study aimed to investigate the clinical, pathological, and genetic characteristics and prognosis of RR-MADD to improve the understanding of clinicians and provide a theoretical basis for the early diagnosis and treatment of RR-MADD.

MATERIAL AND METHODS

Patients

From January 2005 to November 2020, 31 patients were admitted to the Department of Neurology due to muscle diseases and suspected of LSMs pathologically, which were diagnosed as RR-MADD based on gene results and riboflavin treatment effect. The improvement...
of effect was made based on clinical recovery including the disappearance of muscle weakness, exercise intolerance, and pathological recovery including the disappearance of abnormal fat deposition in muscle fibers after riboflavin treatment. This study was approved by the ethical committee of the local hospital, and informed consent was obtained from each patient. Muscle and/or blood samples were collected from all patients.

Clinical Data
Patients' clinical data were collected, including sex, age at onset, first symptoms, disease course, characteristics of weakness, muscle strength (modified Medical Research Council; 0-5 point manual muscle testing score), other systems involved, laboratory tests, and electromyography. Blood samples from 13 patients and urine samples from 12 patients before riboflavin treatment were collected. Appropriate amounts of peripheral blood and fresh morning urine were analyzed for acylcarnitine and organic acid, respectively, by high-performance liquid chromatography-mass spectrometry and gas chromatography-mass spectrometry. Patients were followed up either by telephone or outpatient visits.

Muscle Biopsy and Muscle Pathology
Muscle biopsy was performed on all patients under local anesthesia. The deltoid muscle, biceps brachii, gastrocnemius muscle, or quadriceps femoris muscle was selected as the surgical site according to the patient’s condition. The removed muscle specimens were embedded and placed in isopentane that was precooled in liquid nitrogen. Frozen muscle specimens were placed in a frozen slicer at approximately -22 °C. Frozen sections with a thickness of 8 μm were stained with hematoxylin-eosin (HE), modified Gomori trichrome (MGT), oil red O (ORO), succinate dehydrogenase (SDH), nicotinamide adenine dinucleotidetetrazolium reductase (NADH-TR), ATPase (pH 4.5, pH 10.2), nerve specific enolase (NSE), and periodic acid Schiff (PAS). The pathological characteristics of each patient were observed and recorded in detail.

Genetic Analysis
Genomic DNA of 1-3 μg was extracted, interrupted, and amplified to establish standard DNA libraries.

The amplified DNA libraries were captured using GenCap skeletal system capture kit (My Genostics, China). The gene panel for skeletal system disease includes 239 genes such as ETFDH, ETFB, FDNB,大盘, PMLA2. High-throughput sequencing was performed using the sequencer HiSeq 2000 (Illumina, CA, USA). Single nucleotide polymorphism or insertion/deletion (Indel) can be analyzed by bioinformatics to identify the mutation information of related genes. According to the results of exome capture and sequencing, Sanger sequencing was performed to verify the possible pathogenic gene mutations.

RESULTS
Clinical Data Results
In this study, 18 male and 13 female patients were enrolled. The age at onset ranged from 9 to 59 years. This disease was mainly characterized by a chronic course and fluctuating symptoms. The most common clinical features in all patients were symmetrical muscle weakness, mainly proximal muscle weakness. Eight patients developed neck muscle weakness. Fourteen patients had fluctuating symptoms during the disease course. Some environmental factors, such as cold, infection, and fatigue, can make the disease deteriorate. Nine patients had gastrointestinal symptoms, including nausea, vomiting, and diarrhea (Table 1).

Laboratory examination revealed an elevated plasma creatine kinase level ranging from several to 194 times the normal value in 24 patients. Twelve patients had elevated homocysteine levels, and 12 patients had elevated uric acid levels. Electromyography showed diversiform changes including myogenic (15 of 26), neurogenic and myogenic (5 of 26), neurogenic (2 of 26), and normal (4 of 26) patterns (Table 1). Acylcarnitine analysis of 13 samples showed a combined elevation of medium-chain (C6, C8, C10, and C12) and long-chain (C14 and C16) acylcarnitines in nine patients. Organic acid analysis of 12 urine samples revealed an increase in various dicarboxylic acids (glutarate, adipate, etc.), ethylmalonic acid, and pyruvic acid.

Histopathological Results
Fine vacuoles were observed in muscle fibers among the 31 patients, and some of them fused to form cracks. Atypical ragged red fibers were observed in six patients. The ORO staining showed that lipid droplets were prominently increased in all patients. ATPase staining showed a mosaic distribution of two types of fibers in 31 patients, with vacuolar muscle fibers predominantly in type I fibers (Figure 1).

Genetic Results
Of the 31 patients with RR-MADD, 29 were confirmed to have ETFDH mutations, including homozygous mutations, 19 compound heterozygous mutations, 7 heterozygous mutations, and 2 heterozygous mutations in both ETFDH and ETFA. Two patients had no skeletal system disease panel gene mutation. In this study, a total of 26 ETFDH mutations (NM_004453.4), including 10 novel mutations [c.1534G>A (p.G512R), c. 1552 C>G (p.L518V), c.1285+2T>C, exon 1-5 deletion, c.470C>T (p.P157L), c.511A>G (p.N171D), c.1819dupG (p. E607Gfs* 16), exon 1-6 deletion, c.1085_1107del (p. A363Lfs*18)] and a ETF4 novel mutation (NM_000126.4: c.796A>G (p.T266A)) were found. There are two frequent mutations: c.770A>G (p.Y257C) mutation and c.796A>G (p.T266A) mutation found in six different chromosomes and c.1227A>C (p.L409F) mutation found in nine different chromosomes. The mutation types include missense, splicing, and frameshift mutations, and most of them are missense mutations. The mutation sites of ETFDH and ETFA are summarized in Table 2.

Treatment and Prognosis
All patients received riboflavin, energy support, and other drug therapy, and the response was good. The clinical symptoms began to improve after 1-2 weeks of treatment. Patient 17 regained nearly normal muscle strength after riboflavin treatment. Re-examination of muscle pathology showed that the amount of fat drops

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| N  | Sex | Onset age (year) | Course (year) | Initial symptom | Exercise intolerance | Gastrointestinal symptom | Neck muscle weakness | Chewing weakness | Dysphagia | Respiratory muscle weakness | Symmetry (yes/no) | Upper limb | Lower limb | CK | HCY | UA | EMG |
|----|-----|------------------|---------------|-----------------|---------------------|--------------------------|---------------------|-----------------|-----------|-----------------------------|------------------|------------|------------|----|----|----|-----|
| 1  | M   | 16               | 0.58          | B-WLL           | +                   |                          | Y                   | 5               | 5          | 5-            | 5               | 1192       | -          | 523 | MD  |
| 2  | M   | 21               | 1             | B-WLL           | Y                   |                          | 4                   | 5               | 5          | 5-            | 5               | 2250       | -          | 587 | MD  |
| 3  | F   | 39               | 13            | WL              | Y                   |                          | 4                   | 5               | 4          | 4              | 226             | -          | 178.8     | NCAG |
| 4  | F   | -                | -             | -               | -                   |                          | -                   | -               | -          | -              | -                | -          | -         | ND  |
| 5  | M   | 10               | 4             | WL              | Y                   |                          | 5                   | 5               | 5-          | 5              | 10520           | -          | -         | ND  |
| 6  | M   | 13               | 1             | B-WLL           | Y                   |                          | 4                   | 5               | 3          | 5              | 15050           | -          | 1516.2    | MD+ |
| 7  | F   | 47               | 1             | B-WUL           | +                   |                          | +                   | +               | +          | +              | +                | -          | -         | ND  |
| 8  | F   | 59               | 0.08          | WL              | +                   |                          | +                   | +               | +          | +              | +                | -          | -         | ND  |
| 9  | M   | 25               | 1             | WL              | Y                   |                          | 5                   | 5               | 5          | 5              | 620             | 33.7       | 537       | ND  |
| 10 | M   | 25               | 0.33          | WL              | +                   |                          | 4                   | 5               | 4          | 5              | 108             | -          | 554.2     | NCAG |
| 11 | F   | 9                | 6             | WL              | +                   | +                       | 3                   | 4               | 3          | 4              | 410             | 15.1       | 436.3     | MD  |
| 12 | F   | 30               | 0.5           | B-WLL           | +                   |                          | Y                   | 4               | 5          | 4              | 5               | 375        | 25.9      | 489 | MD+|
| 13 | M   | -                | -             | -               | -                   |                          | -                   | -               | -          | -              | -                | -          | -         | ND  |
| 14 | M   | -                | -             | -               | -                   |                          | -                   | -               | -          | -              | -                | -          | -         | ND  |
| 15 | M   | 37               | 1             | WL              | +                   |                          | Y                   | 5               | 5          | 5-            | 5               | 394        | -         | -   | NCAG|
| 16 | F   | 30               | 0.5           | WL              | +                   |                          | Y                   | 5               | 5          | 5              | 4               | 1971       | 158.7     | 135 | ND  |
| 17 | F   | 17               | 0.5           | WL              | +                   |                          | Y                   | 5               | 5          | 5              | 5               | 8234       | -         | -   | MD  |
| 18 | M   | 19               | 0.33          | B-WLL           | +                   |                          | Y                   | 5               | 5          | 5-            | 5               | 22842      | 58        | 361 | NCAG|
| 19 | F   | 47               | 0.08          | B-WLL           | +                   |                          | Y                   | 5               | 5          | 5-            | 5               | 89.1       | 11.1      | 167.2 | MD  |
| 20 | M   | 57               | 0.08          | B-WLL           | +                   |                          | Y                   | 5               | 5          | 5              | 5               | 1043       | -         | -   | MD  |
| 21 | M   | 29               | 0.5           | WL              | +                   |                          | Y                   | 4               | 5          | 5-            | 5               | 1429       | -         | 386 | MD  |
| 22 | M   | 29               | 0.02          | WL              | +                   |                          | Y                   | 4               | 5          | 4              | 5               | 2649       | -         | 755  | MD  |
| 23 | M   | 34               | 0.83          | WL              | +                   |                          | Y                   | 5               | 5          | 5              | 5               | 571        | 10.8      | 406  | MD  |
| 24 | M   | 23               | 6             | B-WLL           | +                   |                          | Y                   | 5               | 5          | 5              | 5               | 537        | 7.7       | 761.5 | MD+|
| 25 | M   | 28               | 0.08          | B-WLL           | +                   |                          | Y                   | 5               | 5          | 5-            | 5               | 147        | 83.1      | 381  | -   |
| 26 | M   | 58               | 4             | B-WLL           | +                   |                          | Y                   | 5               | 5          | 5-            | 5               | 2419       | 29.1      | 371  | MD  |
| 27 | F   | 40               | 1             | WL              | +                   | +                       | Y                   | 4               | 5          | 3              | 5               | 1581.1     | 29.1      | 707  | MD  |
| 28 | F   | 43               | 3             | WL              | +                   | +                       | Y                   | 5               | 5          | 3              | 5               | 881        | 8.5       | 278  | MD+|
| 29 | F   | 20               | 5             | WL              | +                   | +                       | +                   | 4               | 5          | 4              | 5               | 425        | 22.6      | 313  | MD  |
| 30 | F   | 34               | 3             | WL              | +                   | +                       | +                   | 3               | 4          | 3              | 4               | 4302       | 33        | 501  | MD  |
| 31 | M   | 35               | 0.42          | WL              | +                   | +                       | +                   | +               | +          | +              | +                | 60352      | 61.8      | 554  | MD  |

M, Male; F, female; WL, weakness of limbs; B-WLL, bilateral weakness of lower limbs; B-WUL, bilateral weakness of upper limbs; P, proximal; D, distal; CK, creatine kinase (reference values, 50-310 U/L); HCY, homocysteine (reference values, 0-15 μmol/L); UA, uric acid (reference values, 208-428 μmol/L); EMG, electromyography; MD, myogenic damage; ND, neurogenic damage; NCAG, nothing abnormal detected; -, not provided. Muscle strength was assessed according to a modified Medical Research Council muscle grading system.
FIG. 1. Pathological characteristics of the muscle tissue.

a) Hematoxylin and eosin (HE) staining (×400) of normal controls; b) HE staining (×400) showed vacuolar muscle fibers and atrophic muscle fibers (patient 27); c) oil red O (ORO) staining (×400) of normal control; d) ORO staining (×400) showed lipid deposition between muscle fibers (patient 27); e) modified Gomori trichrome staining (×400) showed ragged red fibers (patient 15); f) ATPase (PH 10.2) staining (×400) showed that the vacuolar muscle fibers were mainly type I fibers (patient 31).
TABLE 2. Summary of Mutations in RR-MADD Related Genes

| N  | Gene     | Exon  | Nucleotide | Amino acid | Homozygous/heterozygous |
|----|----------|-------|------------|------------|--------------------------|
| 1  | ETFDH    | Exon3 | c.250G>A   | p. A84T    | Het                      |
|    | ETFDH    | Exon12| c.1531G>A  | p. D511N   | Het                      |
| 2  | ETFDH    | Exon7 | c.770A>G   | p. Y257C   | Het                      |
|    | ETFDH    | Exon12| c.1534G>A  | p. G512R   | Het                      |
|    | ETFDH    | Exon12| c.1552C>G  | p. L518    | Het                      |
| 3  | ETFDH    | Exon7 | c.770A>G   | p. Y257C   | Het                      |
|    | ETFDH    | Exon12| c.1531G>A  | p. D511N   | Het                      |
| 4  | ETFDH    | Exon3 | c.1227A>C  | p. L409F   | Het                      |
|    | ETFDH    | Exon3 | c.1227A>C  | p. L409F   | Het                      |
|    | ETFDH    | Exon11| c.1395T>G  | p. Y465X   | Het                      |
|    | ETFDH    | Exon5 | c.524G>A   | p. R175H   | Het                      |
| 8  | ETFDH    | Exon3 | c.389A>T   | p. D130V   | Het                      |
|    | ETFDH    | Exon10| c.1227A>C  | p. L409F   | Het                      |
| 9  | ETFDH    | Exon3 | c.389A>T   | p. D130V   | Het                      |
|    | ETFDH*   | Exon10| c.1285+2T>C| splicing   | Het                      |
| 10 | ETFDH    | Exon10| c.1211T>C  | p. M404T   | Het                      |
|    | ETFDH*   | Exon1-5| deletion   |            |                           |
| 11 | ETFDH    | Exon3 | c.250G>A   | p. A84T    | Het                      |
|    | ETFDH    | Exon7 | c.770A>G   | p. Y257C   | Het                      |
|    | ETFDH    | Exon12| c.1534G>A  | p. G512R   | Het                      |
| 12 | ETFDH    | Exon3 | c.250G>A   | p. A84T    | Het                      |
|    | ETFDH    | Exon5 | c.511A>G   | p. N171D   | Het                      |
| 13 | ETFDH*   | Exon9 | c.796A>G   | p. T266A   | Het                      |
|    | ETFDH    | Exon10| c.1285+1G>A| splicing   | Het                      |
| 14 | ETFA*    | Exon9 | c.796A>G   | p. T266A   | Het                      |
|    | ETFDH*   | Exon10| c.1285+1G>A| splicing   | Het                      |
| 15 | –        |       |            |            |                          |
| 16 | ETFDH    | Exon13| c.1691-3C>G| splicing   | Het                      |
| 17 | ETFDH*   | Exon4 | c.470C>T   | p. P157L   | Het                      |
|    | ETFDH    | Exon7 | c.770A>G   | p. Y257C   | Het                      |
| 18 | ETFDH    | Exon7 | c.770A>G   | p. Y257C   | Het                      |
|    | ETFDH    | Exon10| c.1227A>C  | p. L409F   | Het                      |
| 19 | –        |       |            |            |                          |
| 20 | ETFDH*   | Exon13| c.1819dupG| p. E607Gfs*16| Het|                |
| 21 | ETFDH    | Exon12| c.1531G>A  | p. D511N   | Het                      |
| 22 | ETFDH    | Exon10| c.1227A>C  | p. L409F   | Het                      |
deposited in muscle fibers was significantly reduced. All patients were followed up for a maximum of 10 years, of which nine patients only had follow-up data during hospitalization due to loss of contact. The muscle strength completely returned to normal in 15 patients, and seven patients were weak compared with healthy ones. Symptoms of limb weakness recurred in four patients, mostly due to fatigue. After treatment with riboflavin, the symptoms were relieved. Eight patients did not take riboflavin after symptom improvement.

**DISCUSSION**

MADD phenotypes are divided into three categories: a neonatal-onset form with congenital anomalies (type I) or without congenital anomalies (type II) and a mild and/or late-onset form (type III). Neonatal-onset forms are usually fatal and characterized by metabolic disorders and hypotonia. Late-onset cases are common, with onset age ranging from adolescence to old age. Patients with late-onset forms often have symptoms of muscle involvement, mostly characterized by fluctuating or progressive muscle weakness and exercise intolerance. All patients in this group had late-onset MADD. The clinical symptoms in this group improved after treatment with riboflavin; thus, cases were referred to as RR-MADD. In this study, the incidence rate of patients by onset age and onset characteristics were basically consistent with previous domestic reports on LSMs. Patients mainly manifested symmetrical limb weakness, partly manifested as neck weakness and respiratory muscle weakness, whereas some patients have induced factors, including cold, infection, and fatigue. The clinical phenotype of late-onset MADD may be influenced by environmental factors. Patients can have multiple system involvement, and nine patients also demonstrated digestive symptoms, including nausea, vomiting, and diarrhea, which should arouse the warning of clinicians.

Muscle damage can lead to an increase in creatine kinase levels. Mild-to-moderate elevation of creatine kinase occurred in most patients. Electromyography is of great value in the diagnosis of muscle diseases. Although RR-MADD is a muscle disease, its electromyography lacks corresponding specificity. It can be manifested as myogenic damage, neurogenic damage, or normal findings. In this study, electromyography manifestations were mainly myogenic damage, which was consistent with previous reports. Studies have found that the screening of acylcarnitine and organic acid in patients with LSMs can be helpful for the classification and diagnosis. Elevated levels of acylcarnitines and organic acid were observed in the patients. The detection of acylcarnitines and organic acids may be interfered with by several factors, such as drugs, diet, and disease stage. Therefore, the value of screening acylcarnitine and organic acid alone is limited; thus, it should be combined with clinical manifestations and genetic testing for comprehensive analysis. Muscle biopsy and pathology are of great significance in the diagnosis of LSMs. Muscle biopsy of patients presented with typical pathological changes like lipid deposition, mainly involving type I fibers. In this study, several scattered small round vacuoles were observed in the muscle fibers of patients by hematoxylin and eosin (HE) staining. ORO staining showed fatty deposition with type I muscle fibers. In addition, results of MGT, SDH, NADH-TR, ATPase (pH 4.5, pH 10.2), NSE, and PAS staining were consistent with pathological changes in

| N  | Gene | Exon   | Nucleotide     | Amino acid | Homozygous/heterozygous |
|----|------|--------|----------------|------------|-------------------------|
| 23 | ETFDH| Exon10 | c.1227A>C      | p. L409F   | Het                     |
|    | ETFDH| Exon1-6| deletion       |            | Hete                    |
| 24 | ETFDH| Exon7  | c.770A>G       | p. Y257C   | Hemi                    |
|    | ETFDH| Exon7  | deletion       |            | Hete                    |
| 25 | ETFDH| Exon10 | c.1227A>C      | p. L409F   | Hete                    |
| 26 | ETFDH| Exon7  | c.770A>G       | p. Y257C   | Hom                     |
| 27 | ETFDH| Exon7  | c.770A>G       | p. Y257C   | Hete                    |
| 28 | ETFDH| Exon9  | c.1085_1107del | p. A363Lfs*18 | Hete                  |
| 29 | ETFDH| Exon1  | c.3G>C         | p. M1I     | Hete                    |
| 30 | ETFDH| Exon3  | c.389A>T       | p. D130V   | Hete                    |
| 31 | ETFDH| Exon11 | c.1395T>G      | p. Y465X   | Hete                    |
|    | ETFDH| Exon7  | c.770A>G       | p. Y257C   | Hete                    |
|    | ETFDH| Exon11 | c.1450T>C      | p. W484R   | Hete                    |
|    | ETFDH| Exon2  | c.65A>G        | p. K22R    | Hete                    |

Het, heterozygous; hom, homozygous; hemi, hemizygous; *, novel mutations; −, not found.
LSMs. Genetic testing is also of great significance in the diagnosis of RR-MADD. A study reported that ETFDH mutations are major causes of RR-MADD. Through genetic testing, most of the cases were related to ETFDH mutations. MADD is an autosomal recessive disease that can be caused by compound heterozygous or homozygous mutations. However, in this study, nine patients with RR-MADD were found to carry a heterozygous ETFDH mutation, and clinical manifestations were improved with the treatment of riboflavin. However, further investigations are needed to analyze the possible reasons and the mutation sites located in regions outside the exon that cannot be identified by genomic sequencing. In addition, two patients had no pathogenic gene mutations, which were clinically and pathologically diagnosed with RR-MADD. They had symptoms of muscle weakness and presented with typical pathological changes such as lipid deposition. The symptoms were improved after treatment with riboflavin. During the follow-up period, muscle weakness recurred due to fatigue, but improved after treatment with riboflavin again. Further study is needed to determine whether unknown gene mutations are at play.

Flavin adenine dinucleotide (FAD) is the cofactor of ETF or ETF-QO and has been demonstrated to promote folding and conformational stability and activity of protein. Riboflavin is the precursor of FAD, and riboflavin supplementation can increase cellular FAD content and thus improve clinical symptoms. However, the specific application period and maintenance dose of riboflavin, influencing factors of the fluctuation of RR-MADD symptoms during treatment, and characteristics of clinical outcomes still need to be further studied in a larger sample size. In this study, the riboflavin dosage in the patients ranged from 15 to 210 mg/dl. Riboflavin is not stored in the body, and intakes of riboflavin above tissue requirements are excreted in the urine as riboflavin or other metabolites. Alternatively, an increase in FAD-binding flavoproteins after riboflavin supplementation could release more FAD to the mitochondrial matrix during degradation, keeping a larger circulating FAD pool even after discontinuation of riboflavin treatment. Eight patients in this study did not take riboflavin after symptom improvement. For some patients, short-term riboflavin supplementation brings a long period of symptom resolution. The clinical manifestations and genetic test results of patients with RR-MADD were heterogeneous. Therefore, a comprehensive analysis of clinical, pathological, and genetic testing is essential for the early diagnosis of RR-MADD. In addition, when early diagnosis is difficult, experimental drug therapy can be considered to observe its efficacy.

Ethics Committee Approval: This study was approved by the ethical committee of the local hospital.

Informed Consent: Written informed consent was obtained from each patient.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authorship Contributions: Design- Y.S., X.S.; Data Collection or Processing- Y.W., Y.Wu.; Analysis or Interpretation- G.J.; Literature Search- L.X., L.M.; Writing- J.Z., J.H.;

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