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Original Article

Mitochondrial genetic analysis in a Chinese family suffering from both mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes and diabetes

Weiwei Li*, Wei Zhang*, Fang Li, Cailing Wang

*Department of Endocrinology, Qianfoshan Hospital, Shandong University, 66 Jingshi Road, Jinan 250014, China. *Equal contributors.*

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Abstract: To investigate the mitochondrial mutations in patients suffering from both mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) and maternally inherited diabetes. MELAS was confirmed by muscle biopsy performed from the biceps muscle of the proband. Mitochondrial DNA (mtDNA) was isolated from peripheral blood mononuclear cells. The significant mtDNA loci of other 14 family members were further detected according to the sequencing results of the proband. Direct sequencing of PCR products was used to identify the mitochondrial mutations. The proband (III 1) and her brother (III 3) both harbored the tRNALeu (UUR) A3243G mutation, with heteroplasmic levels of 50% and 33% respectively. Moreover, another two mitochondrial variants, A8860G and A15326G, were also detected in the samples of all the family members. MELAS and diabetes can coexist in one patient, and the main cause for these diseases is the tRNALeu (UUR) A3243G mutation. However, other gene variants may contribute to its pathogenesis. This case also supports the concept that both syndromes can be regarded as two phenotypes of the same disease.

Keywords: Diabetes, A3243G mutation, MELAS, heteroplasmy, polymorphism, mitochondrial myopathy

Introduction

Mutations in mitochondrial DNA (mtDNA) have been proved to be associated with a wide spectrum of clinical abnormalities, including neuromuscular disorders, cardiomyopathy, end-stage renal failure, diabetes, hearing and visual loss [1]. In particular, the A3243G mutation in tRNALeu (UUR) is considered to be the most common pathogenic mutation in mtDNA, which may cause many abnormalities, such as MELAS, and maternally inherited diabetes and deafness (MIDD) [2]. MIDD accounts for 0.5-3% of diabetes and is characterized as decreased insulin secretion and sensorineural hearing loss [3]. On the contrary, MELAS is a more severe syndrome, which features in stroke-like episodes, encephalopathy, myopathy and lactic acidosis [4]. Patients with different clinical features may be due to the different levels of heteroplasmy, i.e, the presence of a mixture of mutant and normal mtDNA in one cell [5].

In 2011, a female aged 23 at that time was admitted to our hospital. She was diagnosed with diabetes and oral antihyperglycemic agents were initiated. However, the glycemic control was unsatisfactory, so insulin treatment was prescribed soon. Moreover, she gradually developed episodic headaches, nausea, vomiting and seizures. At the age of 25, headache and seizures worsened and occurred more frequently. On careful inquiry of her family history, we discovered that both her grandmother and mother had diabetes. We then suspected that the pedigree's diabetes might be due to mtDNA mutations. In this research, we report the clinical and pathological features of the patient who finally been diagnosed as both MELAS and diabetes, as well as the mtDNA mutations in her whole family.

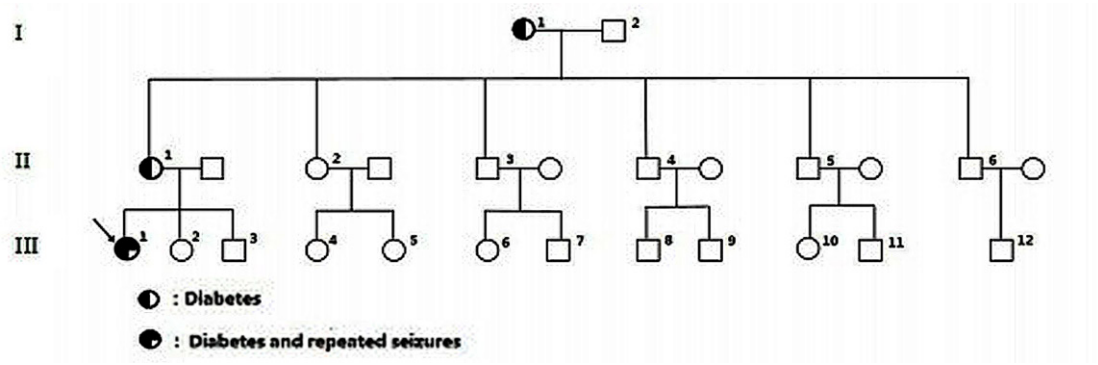

Figure 1. Pedigree of the family with diabetes and seizures. Arrow marks the proband.

Table 1. The blood test results of the proband

Items	Result	Reference value
FPG	9.1 mmol/l	3.89-6.11 mmol/l
HbA_1C	8.7%	4-6%
IAA	2.93 iu/ml	0.41-20 iu/ml
GAD	9.88 iu/ml	0.51-30 iu/ml
lactate	4.06 mmol/l	1.32-1.76 mmol/l

Materials and methods

Muscle biopsy

To confirm the disease status, an open biopsy was performed from the biceps muscle of the proband in accordance with standard operating procedures. All the processes including the following steps were approved by the Ethics Committee of the Shandong Provincial Qianfoshan Hospital. All participants provided their written informed consent to participate in this study. The biopsy specimens were prepared for histopathological studies, including staining with hematoxylin and eosin (H&E), modified Gomori trichrome (MGT), nicotinamide adenine dinucleotide (NADH) tetrazolium reductase, succinate dehydrogenase (SDH), cytochrome C oxidase (COX), periodic acid Schiffs (PAS), and oil red O staining (ORO).

Mitochondrial DNA sequencing and mutation analysis

We firstly sequenced the whole mtDNA genome of the proband, and then detected the significant mtDNA loci of other 14 family members according to the sequencing results of the proband. Informed consent of this study was obtained from all who enrolled in the study. All the samples were obtained from peripheral blood mononuclear cells. The PCR reaction was performed in the following conditions: 95°C for 3 min followed by 30 cycles (95°C for 30 s, 56°C for 30 s, and 72°C for 45 s) and a final extension at 72°C for 10 min in a thermal cycler. Each PCR product was purified and then sequenced in an automated DNA sequencer as described in our previous study [6]. These sequence results were aligned with the revised Cambridge Reference Sequence (rCRS) (NC-012920).

Results

Pedigree and clinical features

The proband (III 1 in **Figure 1**) was a 25-year-old female, with a BMI of 17.31 kg/m^2 (height 152 cm, weight 40 kg). Since the effect of oral antihyperglycemic agents was poor, insulin therapy was prescribed soon after the diagnosis of diabetes and the total insulin dose was 20 IU/d. Furthermore, she developed repeated headache, nausea, vomiting and seizures, although without obvious deafness. Blood test results were summarized in **Table 1**. The proband's grandmother (I 1 in **Figure 1**) had late-onset diabetes and was receiving 250 mg metformin once a day. The patient's mother (II1) was diagnosed with diabetes 2 years ago, and she also had abnormal level of lactic acid (3.63 mmol/L), but she did not take any medical treatment. None of them had deafness, headache, nausea or seizures. The proband's aunt (II 2), uncles (II 3- II 6), cousins (4- III 11), sister (III 2) and brother (III 3) had no signs of diabetes, hearing disturbances, abnormal levels of lactic acid, stroke-like episodes, cardiac abnormalities, or other severe symptoms.

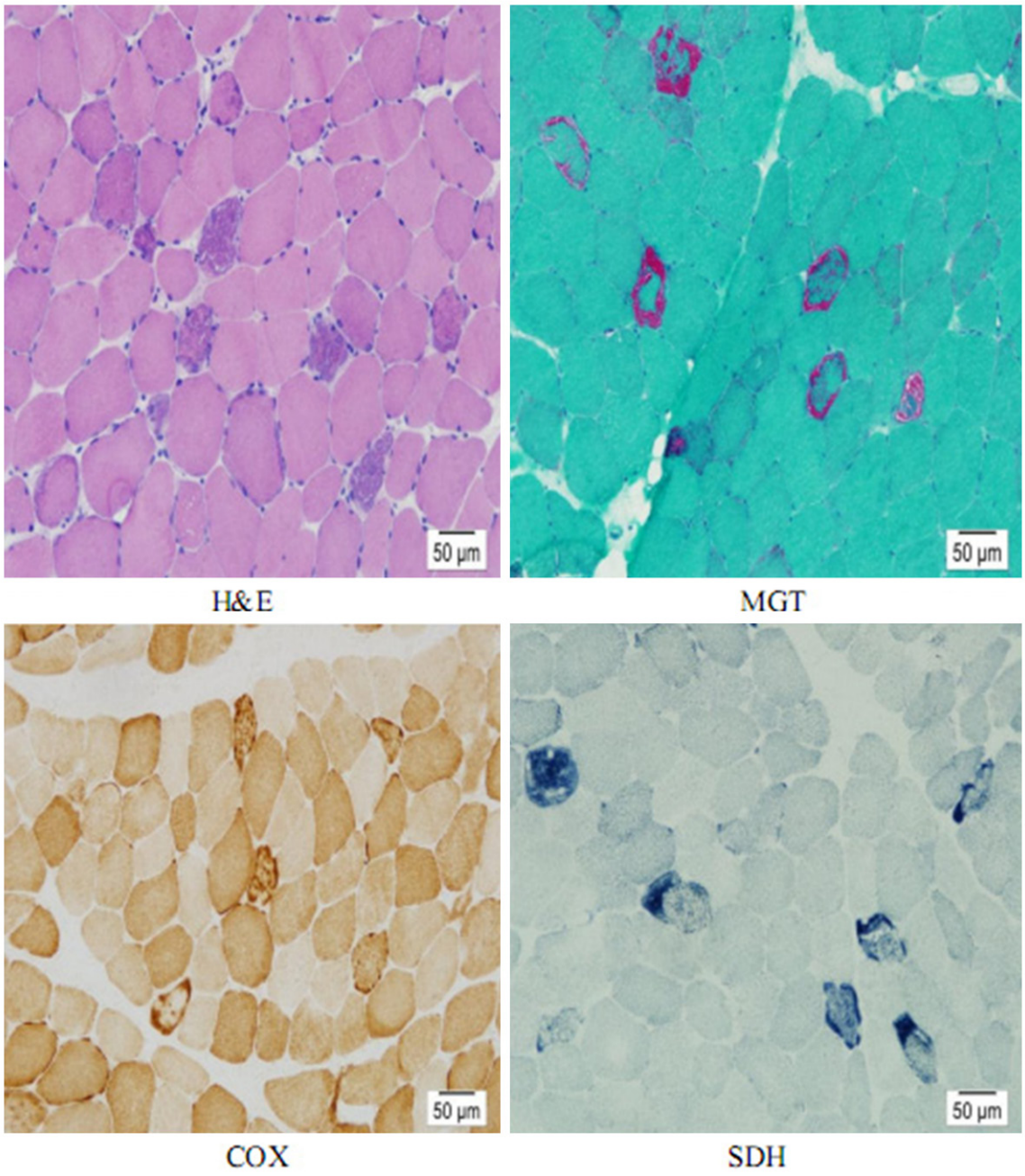

Figure 2. The muscle pathological staining of the proband.

Confirmation of mitochondrial myopathy

On microscopic examination (**Figure 2**), muscle biopsy samples showed marked fiber size variation. We detected ragged-red fibers (15%) in the MGT staining, and several COX-negative fibers in the COX activity staining. In addition, the SDH activity staining showed one strongly SDH-reactive blood vessel. Ragged-red fibers accounting for over 15% of all fibers were defined as mitochondrial myopathy [7]. (Neuromuscular Pathology Laboratory, Qilu Hospital of Shandong University, Pathological number: 10-121).

Determination of mtDNA mutations

The proband (III 1) and her brother (III 3) both harbored the tRNALeu(UUR) A3243G mutation, while other maternal members did not (**Figure**

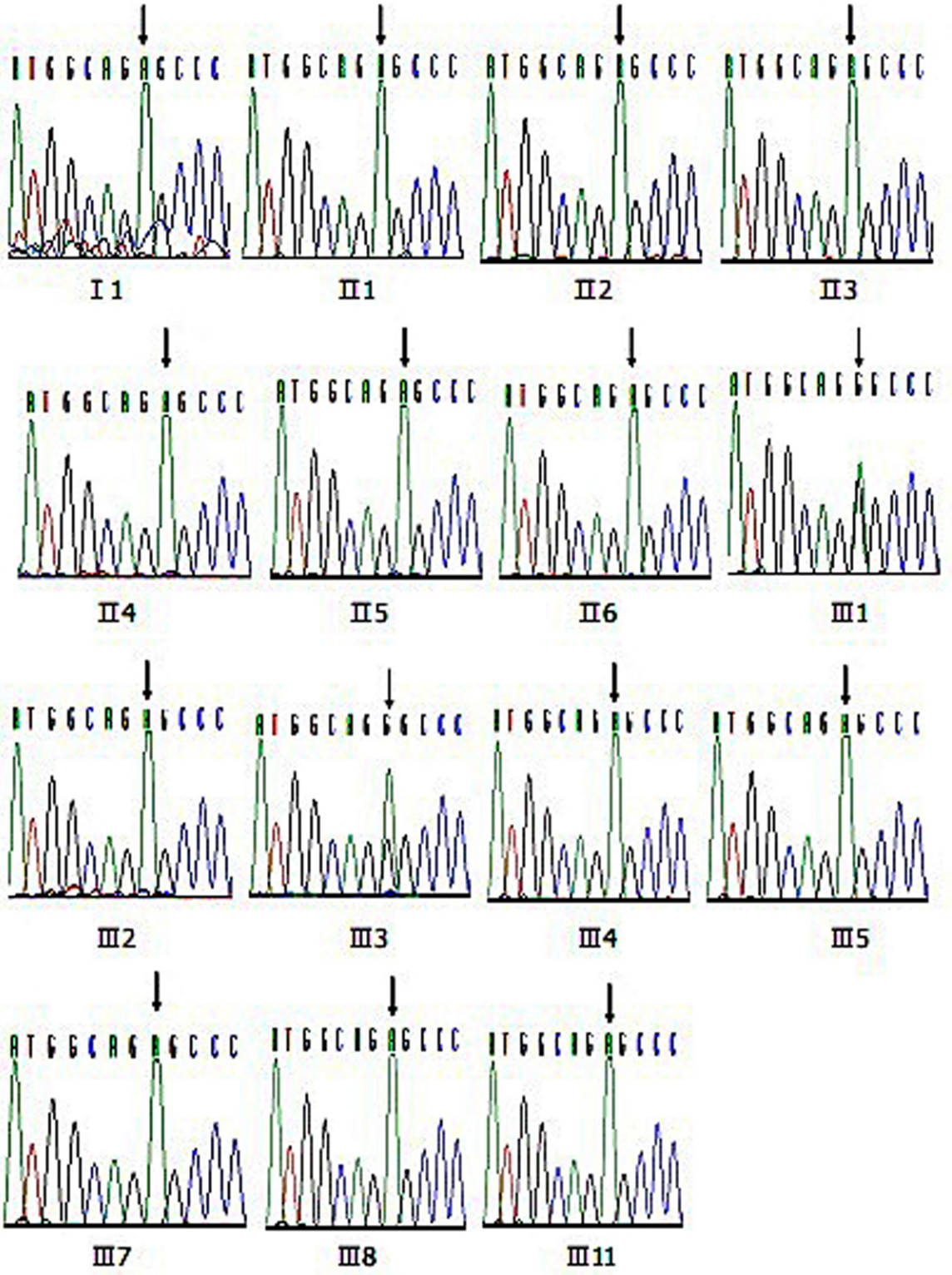

Figure 3. The mitochondrial tRNALeu (UUR) A3243G mutation is present in the proband and her brother (III 1 and III 3), but not found in other maternal members. Arrows indicate nucleotide position 3243.

3). Moreover, we found three other significative mitochondrial variants, A4769G, A8860G and A15326G mutations, from samples of all the 15 family members. All sequencing differences

Table 2. Differences in mitochondrial DNA between the patient and the revised Cambridge reference sequence

Locus name	gene mutation	Mutation type	AA change	Associated disease
D-loop	93	A to G	Non-coding	polymorphism
	152	T to C	Non-coding	polymorphism
	263	A to G	Non-coding	polymorphism
	16519	T to C	Non-coding	polymorphism
12s rRNA	750	A to G	Non-coding	polymorphism
	1438	A to G	Non-coding	polymorphism
16s rRNA	2772	C to T	Non-coding	polymorphism
ND2	4769	A to G	Syn(M)	polymorphism
atp6	8860	A to G	T to A	polymorphism
cytb	15326	A to G	T to A	polymorphism
tRNALeu (UUR)	3243	A to G	Non-coding	diabetes

in mtDNA between the proband and the revised Cambridge Reference Sequence were shown in **Table 2**.

Discussion

In this study, we reported a patient who suffered diabetes, episodic headaches, nausea, vomiting, seizures, as well as increased lactic acid level. These symptoms, in addition to maternal inheritance of diabetes, were highly suggestive of a mitochondrial disorder. Besides diabetes, the patient was further confirmed to suffer MELAS by muscle biopsy. Further gene sequencing identified the A3243G point mutation in the mitochondrial MTTL1 gene.

Interestingly, the tRNALeu (UUR) A3243G mutation was detected only in the samples of the proband and her brother. As shown in **Figure 3**, the heteroplasmic level of the proband was approximately 50%, compared with 33% of her brother's, who did not show any symptoms. The explanation for this phenomenon may be dual. Firstly, the mtDNA mutations are mostly heteroplasmic and there is a link between the level of heteroplasmy and the clinical severity of the disease [8]. That is, the higher the heteroplasmy level is, the earlier the age of onset of mitochondrial disease is, and the severer the clinical presentations will be [9]. The majority of individuals may be asymptomatic, because the mtDNA mutation is present at low levels of heteroplasmy or mutation is only pathogenic in the presence of a trigger. In addition, the threshold for mutant load triggering is different according to the type of mitochondrial mutation [10]. Another potential explanation is that the percentage of mutated mitochondria may vary greatly in different tissues [11]. In this study, we assessed mononuclear cells in peripheral blood, which had low and declining heteroplasmy levels upon aging. Studying the heteroplasmy levels in tissues such as muscle, buccal mucosa, kidney and retinal epithelium would be more helpful, but all the participants refused further examination.

In this study, we also found three other genetic variants in all the maternal members. According to the mitochondrial single nucleotide polymorphism (mtSNP) research result, all these three new mutations were polymorphisms: the A4769G mutation was a silent mutation, the A8860G and A15326G mutations had seldomly been reported previously, and they might not greatly affect the function of mitochondria. However, some studies have presented that additional mitochondrial DNA polymorphisms might influence the phenotype of the A3243G mutation [12]. Therefore, further researches are needed for these two mutations.

Several limitations should be noted. In this study, clinical resources were limited, especially in that exclusion of diabetes mellitus was based merely on fasted blood glucose, instead of systemic oral glucose tolerance test (OGTT). Tissues probably with more mutations such as muscle, buccal mucosa, kidney and retinal epithelium were not sampled and tested. We would carry on the follow-up of the family members.

In summary, we demonstrated the diagnosis of the mitochondrial diabetes and MELAS, and

found that the A8860G and A15326G mutations might also be involved in the pathogenesis of mitochondrial disease. Our findings will be helpful for counseling the mtDNA mutations and maternally inherited diseases.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Cailing Wang, Department of Endocrinology, Qianfoshan Hospital, Shandong University, 66 Jingshi Road, Jinan 250014, China. Tel: +86 531 89268735; Fax: +86 531 89268735; E-mail: sdqy.wcl@163.com

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