

# The A to G transition at nt 3243 of the mitochondrial tRNA<sup>Leu(UUR)</sup> may cause an MERRF syndrome

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## Abstract

**Objective**—To verify the phenotype to genotype correlations of mitochondrial DNA (mtDNA) related disorders in an atypical maternally inherited encephalomyopathy.

**Methods**—Neuroradiological, morphological, biochemical, and molecular genetic analyses were performed on the affected members of a pedigree harbouring the heteroplasmic A to G transition at nucleotide 3243 of the mitochondrial tRNA<sup>Leu(UUR)</sup>, which is usually associated with the syndrome of mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS).

**Results**—The proband was affected by a fullblown syndrome of myoclonic epilepsy with ragged red fibres (MERRF), severe brain atrophy, and basal ganglia calcifications, without the MRI T2 hyperintense focal lesions which are pathognomonic of MELAS. Oligosymptomatic relatives were variably affected by lipomas, goitre, brain atrophy, and basal ganglia calcifications. Muscle biopsies in the proband and his mother showed a MELAS-like pattern with cytochrome c oxidase hyperreactive ragged red fibres and strongly succinate dehydrogenase reactive vessels. Quantification of the A3243G mutation disclosed 78% and 70% of mutated mtDNA in the muscle of the severely affected proband and of his oligosymptomatic mother respectively. Nucleotide sequencing of the mitochondrial tRNA<sup>Leu(UUR)</sup> and tRNA<sup>Lys</sup> in the proband's muscle failed to show any additional nucleotide change which could account for the clinical oddity of this pedigree by modulating the expression of the primary pathogenic mutation.

**Conclusion**—So far, MERRF has been associated with mutations of the mitochondrial tRNA<sup>Lys</sup>, and MELAS with mutations of the mitochondrial tRNA<sup>Leu(UUR)</sup>. Now MERRF may also be considered among the clinical syndromes associated with the A to G transition at nt 3243 of the tRNA<sup>Leu(UUR)</sup>.

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Two distinct maternally inherited encephalomyopathies with ragged red fibres have been recognised on clinical grounds: MERRF, which is characterised by myoclonic epilepsy, skeletal myopathy, neural deafness, and optic atrophy,<sup>1</sup> and MELAS, which is defined by stroke-like episodes in young age, episodic headache and vomiting, seizures, dementia, lactic acidosis, skeletal myopathy, and short stature.<sup>2</sup> Molecular genetic studies later confirmed the nosological distinction between the two disorders, showing that MERRF is strictly associated with two mutations of the mitochondrial tRNA<sup>Lys</sup> at nucleotides 8344<sup>3</sup> and 8356,<sup>4</sup> and MELAS with three point mutations of the mitochondrial tRNA<sup>Leu(UUR)</sup> at nucleotides 3243, 3271, and 3291; the 3243 A to G transition (A3243G) is the most common cause of MELAS.<sup>5</sup>

We discuss here the clinical, neuroradiological, morphological, biochemical, and molecular genetic findings in the affected members of a clinically atypical pedigree harbouring the heteroplasmic A3243G "MELAS" mutation.

## Patients and methods

### PEDIGREE ANALYSIS

#### Family member III-1 proband

The proband, an 18 year old man, was the firstborn of non-consanguineous parents (fig 1). Since the age of 5 years, he had had

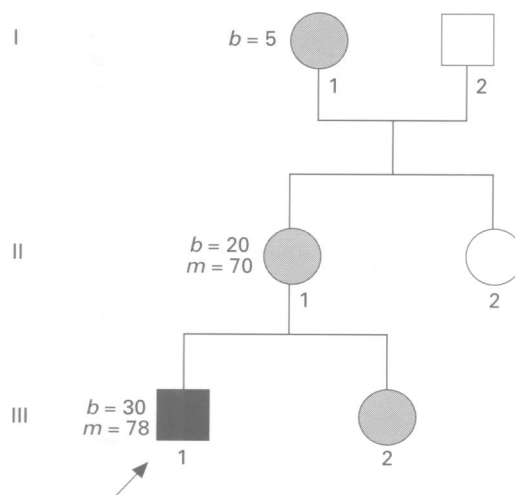


Figure 1 Pedigree: black symbol indicates the severely affected proband; grey symbols indicate the oligosymptomatic members. Percentage of the mutated mtDNA in muscle is given as m; percentage of the mutated mtDNA in blood is given as b.

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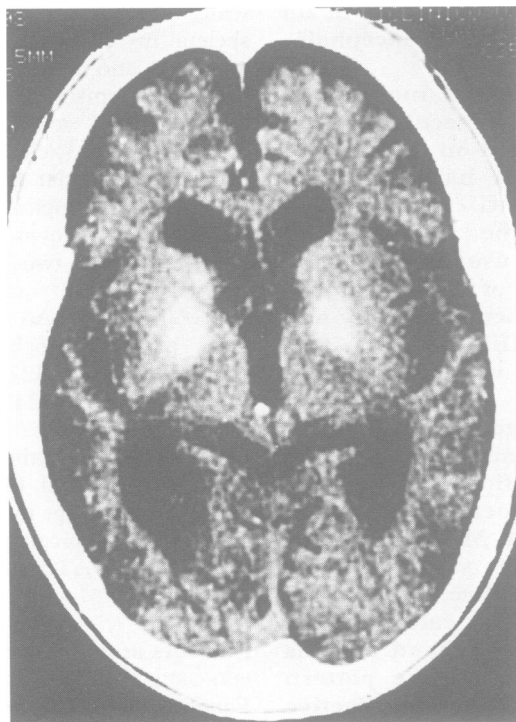
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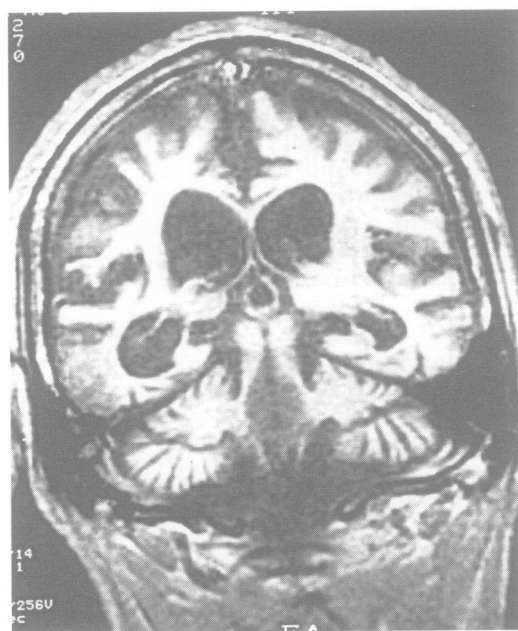
unsteadiness of gait and limb tremors. At the age of 6, he was admitted for generalised seizures; examination disclosed mental retardation, generalised ataxia, intentional tremor and diffuse muscle weakness, and hypotrophy. An EEG showed bilateral irritative elements and generalised slow activity. An EMG had a myopathic pattern in the proximal limb muscles. Brain CT showed bilateral calcifications of the basal ganglia and atrophy of the frontotemporal cortical and subcortical regions (fig 2A). At the age of 7, generalised myoclonic jerks appeared, sometimes followed by grand mal attacks; seizures were frequent (five–10/day) and were partially responsive to val-

proic acid and phenobarbitone. At the age of 10, when the patient was evaluated by us, weight and height were below the 10<sup>o</sup> percentile. His IQ was 62. An ECG showed a block of the right bundle branch; an echocardiogram was normal. There was generalised muscle wasting, gait ataxia and dysmetria, intention myoclonus, nystagmus, hyperreflexia of the lower limbs, bilateral optic atrophy, and cervical lipoma. An EEG showed synchronous bursts of sharp waves and spike wave complexes on the temporal regions. Audiometric tests disclosed neural deafness. Fundoscopy disclosed bilateral optic atrophy with a normal electroretinogram. Proteins in CSF were 103 mg% with a normal IgG/albumin ratio. Abnormal laboratory investigations included blood lactate (40.5 mg/dl, normal 2.7–11.7 mg/dl), pyruvate (1.15 mg/dl, normal 0.26–0.7 mg/dl), and increased lactate to pyruvate ratio. Parathyroid hormone (amino acids 1–84) was 29 pg/ml (normal range 5–10 pg/ml) and calcaemia was 9.1 mg/dl. Brain MRI disclosed diffuse supratentorial and infratentorial atrophy of the cortical and subcortical regions. The syndrome evolved towards a severe mental deterioration with spastic tetraparesis, dysarthria, dysphagia, and recurrent generalised myoclonic seizures. Brain MRI performed at the age of 18 showed a severe atrophy of the cerebral cortex and subcortical white matter, as well as of the semiovale centres and corpus callosum. Cerebellar hemispheres and the vermis were also atrophic (fig 2B).

**Figure 2** (A) Proband's CT (6 years of age) showing calcifications of the basal ganglia and atrophy of the frontotemporal cortical and subcortical regions. (B) proband's T1 weighted MRI coronal section (18 years of age), showing a severe supratentorial and infratentorial cortical and subcortical atrophy.



A



B

#### *Family member II-1: mother of the proband*

The mother of the proband, 43 years old, had a negative neurological history. Since the age of 30 she had had multinodular euthyroid goitre which was treated with thyroxine. Clinical examination disclosed a cervical lipoma. An EMG was normal. Among relevant laboratory investigations, blood lactate, pyruvate, T<sub>3</sub>, T<sub>4</sub>, thyroid stimulating hormone, and parathyroid hormone (amino acids 1–84) were in the normal ranges. Brain CT disclosed bilateral basal ganglia calcifications and atrophy of the frontotemporal cortex, cerebellum, and pons. Ophthalmoscopy and audiometric tests gave normal results.

#### *Non-examined maternal members*

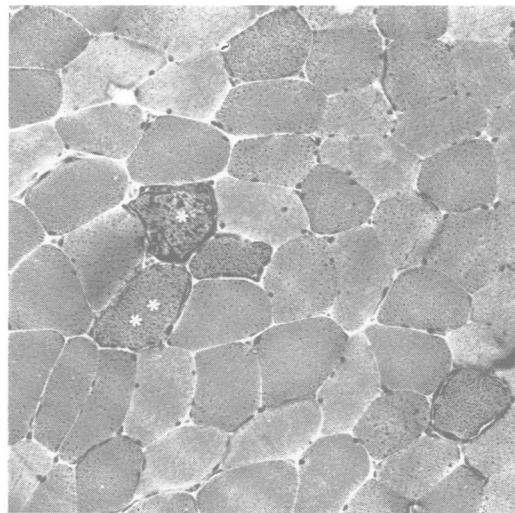
The proband's grandmother, family member I-1, aged 65, had had hyperthyroidism since the age of 25. At the age of 50 fluorangiography had shown bilateral drusen of the optic disc and degeneration of the macular epithelium of the left eye.

The proband's sister, family member III-2, 14 years old, had headache and nasal speech.

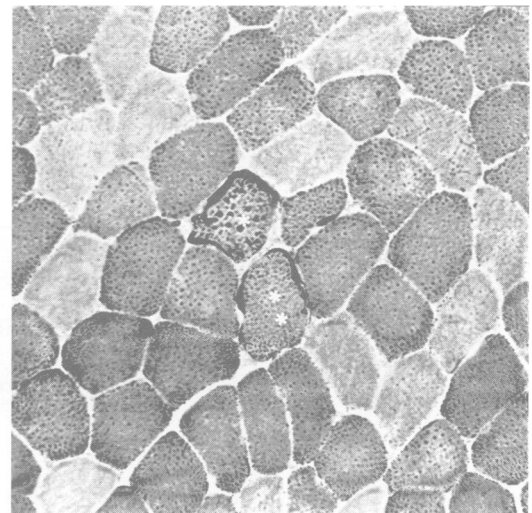
#### MORPHOLOGICAL STUDIES

The proband's biopsy specimens were obtained under local anaesthesia from the right deltoid muscle at the age of 10 and from the left vastus lateralis muscle at the age of 18; a biopsy of the deltoid muscle was also performed in the 43 year old mother. Specimens for histopathological study were frozen in

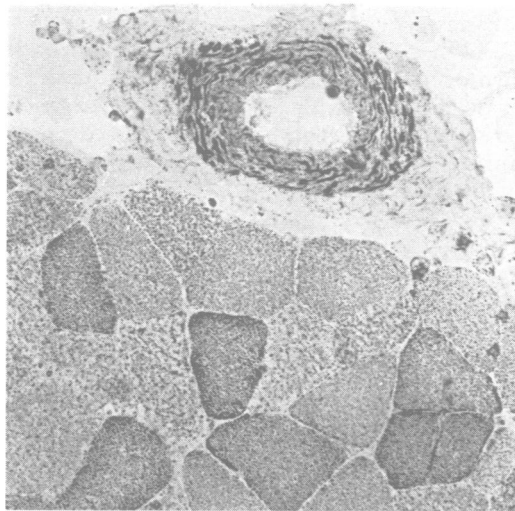
**Figure 3** Modified Gomori trichrome stain (*A* × 140) and cytochrome c oxidase reaction (*B* × 140) in the mother's deltoid muscle; asterisks indicate the corresponding fibres in serial sections. Ragged red fibres have a strong cytochrome c oxidase activity which is more intense at the periphery. (*C*) Succinate dehydrogenase staining on the proband's second muscle biopsy (× 175) shows a small artery with densely stained granules in its wall. (*D*) Electronmicrography of the proband's second muscle biopsy shows aggregates of intermyofibrillar mitochondria containing paracrystalline inclusions (× 4200).



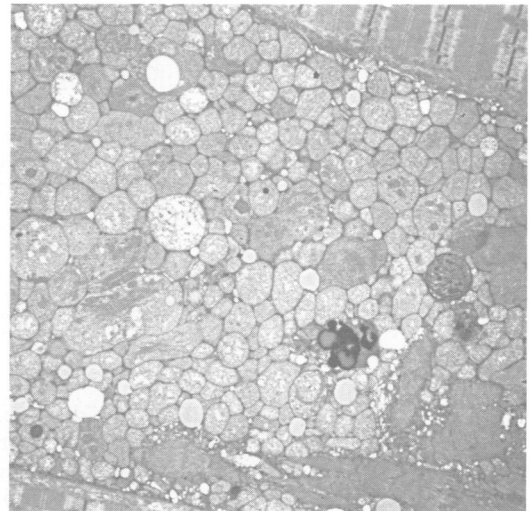
A



B



C



D

isopentane cooled with liquid nitrogen. Serial 8  $\mu\text{m}$  thick transverse sections were stained with haematoxylin and eosin, modified Gomori trichrome, and a battery of histochemical methods including succinate dehydrogenase, routine ATPase, ATPase with preincubation at pH 4.6 and 4.3, and cytochrome c oxidase. Small pieces of the specimens were fixed in 2.5% glutaraldehyde and postfixed in 1% OsO<sub>4</sub> in 0.1 M phosphate buffer. After fixation the specimens were dehydrated and embedded in epoxy resin for electron microscopy

#### BIOCHEMICAL STUDIES

The mitochondrial fraction was isolated from 200–300 mg of frozen muscle samples from family members III-1 and II-1 and the activities of the following respiratory chain enzymes were determined spectrophotometrically as previously described<sup>6</sup>: NADH cytochrome c reductase, succinate cytochrome c reductase, and cytochrome c oxidase. Values were expressed as ratios with citrate synthase, an enzyme of the mitochondrial matrix that is a reliable index of mitochondrial protein content.

#### MOLECULAR GENETIC ANALYSIS

Total DNA was extracted from blood of family members III-1, II-1, and I-1 and from 50 mg of frozen bioptic muscle of members III-1 and II-1, according to standard procedures. The presence of the following pathogenic point mutations of mtDNA were investigated on the proband's muscle mtDNA, by standard RFLP-PCR (polymerase chain reaction) analysis: MERRF A8344G and T8356C at the tRNA<sup>Lys</sup>; MELAS A3243G, T3271C, T3291C at the tRNA<sup>Leu(UUR)</sup>; MELAS T9957C at the COXIII gene; and NARP (neurogenic ataxia and retinitis pigmentosa) T8993G at the ATPase6 gene.

Quantification of the A3243G mutation in blood from I-1, II-1, and III-1 and muscle from II-1 and I-1 was obtained by a routine *ApaI*-RFLP analysis on the PCR amplified mitochondrial tRNA<sup>Leu(UUR)</sup>, modified according to Tanno *et al*<sup>7</sup> to reduce the underestimation of mutated mtDNA caused by the formation of heteroduplexes. Direct sequencing of both strands of the mitochondrial tRNA<sup>Leu(UUR)</sup> and tRNA<sup>Lys</sup> was performed on polymerase chain reaction (PCR) amplified fragments corresponding to nucleotides

## Respiratory chain enzyme activities in skeletal muscle mitochondria

	NADH cytochrome c reductase	Succinate cytochrome c reductase	Cytochrome c oxidase	Citrate synthase
<i>Pedigree members</i>				
III-1	0.15	0.10	0.49	719
II-1	0.19	0.13	0.62	946
controls (n = 50) mean (SD)	0.24 (0.08)	0.19 (0.09)	1.2 (0.45)	690 (229)

Values are nmol/min/mg of mitochondrial proteins.

3150–3369 for the tRNA<sup>Leu(UUR)</sup> and nucleotides 8184–8478 for the tRNA<sup>Lys</sup>, using the "Sequenase PCR product sequencing kit" (Amersham).

### Results

#### MORPHOLOGICAL FINDINGS IN SKELETAL MUSCLE BIOPSIES

The proband's first muscle biopsy (deltoid) showed 8%–10% of ragged red fibres. In the second biopsy (vastus lateralis), ragged red fibres were reduced to 1%–2%, but in many fibres the succinate dehydrogenase staining was stronger than that of normal fibres. The oligosymptomatic mother's muscle biopsy showed 8%–10% of ragged red fibres. Type I fibres were preferentially involved in mitochondrial proliferation. Some ragged red fibres were completely deficient in cytochrome c oxidase, but most showed cytochrome c oxidase hyperreactivity, which was more evident in the periphery of the fibres (fig 3 A,B). Blood vessels stained darkly with succinate dehydrogenase (fig 3C), and were hyperreactive to cytochrome c oxidase. Ultrastructural examination showed intermyofibrillar and subsarcolemmal aggregates of abnormal mitochondria containing paracrystalline inclusions (fig 3D). An increased number of mitochondria were also present in the pericytes of capillaries, endothelial cells, and smooth muscle cells of the small arteries.

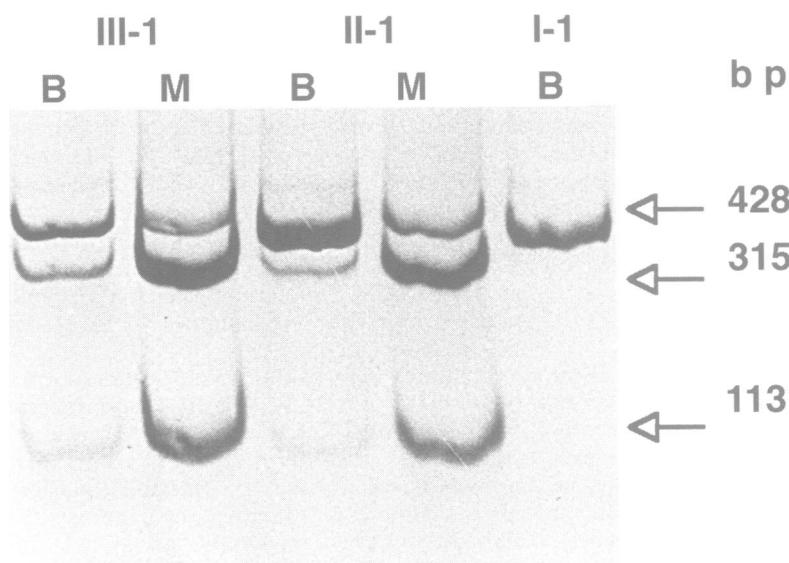


Figure 4 Autoradiogram of PCR products after digestion with *Apa I*. M and B indicate muscle and blood of the examined pedigree members. Wild type mtDNA corresponds to the 428 bp fragment, which is resolved into two fragments of 315 and 113 bp when the A3243G mutation is present. A small percentage (5%) of mutant DNA present in blood from I-1 is not visible on the photographic reproduction.

## ACTIVITIES OF RESPIRATORY CHAIN ENZYMES

Biochemical analysis on the mitochondrial fraction (table) showed a significant decrease of the activities of the NADH cytochrome c reductase (complexes I + III) and cytochrome c oxidase (complex IV) in the skeletal muscle of the proband III-1. Values in the mother's muscle were in the normal range.

#### MOLECULAR ANALYSIS OF MITOCHONDRIAL DNA

PCR-RFLP analysis with *Apa I* showed the heteroplasmic A to G transition at nt 3243 of the mitochondrial tRNA<sup>Leu(UUR)</sup> in the proband's muscle. Quantitative analysis (fig 4) showed that the percentage of the mutated DNA in muscle was 78% in the severely affected proband and 70% in the oligosymptomatic mother. Percentages of mutated DNA in blood were 30%, 20%, and 5% in the proband, mother, and grandmother respectively.

Direct nucleotide sequencing of the proband's muscle mtDNA confirmed the presence of the heteroplasmic A3243G mutation and failed to show any other nucleotide change of the tRNA<sup>Leu(UUR)</sup> and tRNA<sup>Lys</sup>.

### Discussion

The present pedigree harboured the heteroplasmic A to G transition at nucleotide 3243 of the mtDNA, which is typical of MELAS, although the proband was affected by a full-blown MERRF. The syndrome was characterised by seizures, intention myoclonus, ataxia, psychomotor delay, optic atrophy, neural deafness, and cervical lipoma, without any sign or symptom of stroke episodes. A detailed CT and MRI follow up disclosed basal ganglia calcifications and severe, progressive atrophy of the cortex, subcortical white matter, corpus callosum, and semioval centres. Two MRIs, performed at different stages of disease, did not show the T2 hyperintense focal lesions which are pathognomonic of MELAS.<sup>8</sup> Basal ganglia calcifications, although often encountered in MELAS, may be found in various mitochondrial encephalomyopathies, and they are not specific for the disease.<sup>5</sup> The A3243G mutation was first described in MELAS, but it may also occur in association with other two syndromes: chronic progressive external ophthalmoplegia and maternally inherited diabetes with deafness.<sup>9,10</sup> The same mutation has been also identified in a few cases of MERRF/MELAS overlap.<sup>9,14</sup> In these cases, myoclonus or ataxia were always associated with clinical or radiological evidence of stroke-like episodes. More recently, the A3243G mutation was found in a MERRF pedigree,<sup>15</sup> but the occurrence of focal cerebral lesions could not be ruled out in the report because of the absence of MRI studies.

Clinical findings in the oligosymptomatic members of the pedigree also showed atypical features. The proband's mother had cervical lipoma, euthyroid goitre, basal ganglia calcifications, and atrophy of the frontotemporal

cortex and cerebellum. The grandmother had retinopathy and hyperthyroidism. So far, lipomas have been related to the A to G transition at nt 8344 of the tRNA<sup>Lys</sup>,<sup>16,17</sup> in association with MERRF or with Ekbom's syndrome (MERRF plus lipomas, ataxia, and neuropathy), as well as to the autosomal dominant multiple deletions of the mtDNA.<sup>18</sup> Thyroid dysfunction has been reported in a MERRF case<sup>4</sup> and in a MERRF/MELAS pedigree,<sup>19</sup> both associated with a T to C transition at nt 8356 of the tRNA<sup>Lys</sup>. We conclude that lipomas and thyroid dysfunction occur in a wide range of molecular lesions of the mtDNA and they are not specific of any syndrome.

Unlike the clinical phenotype, the morphological abnormalities in muscle followed the pattern seen with the A3243G mutation, which is characterised by ragged red fibres hyperreactive to cytochrome c oxidase and vessels strongly reactive to succinate dehydrogenase.<sup>20</sup>

Quantification of the A3243G mutation showed 78% of mutated muscle mtDNA in the proband and 70% in the oligosymptomatic mother. Percentages of mutated mtDNA in blood were 30%, 20%, and 5% in the proband, mother, and grandmother respectively. Recent surveys showed that the percentages of the A3243G mutation did not correlate with the expression of specific clinical syndromes, although, in the MELAS subgroup, the percentage of mutated mtDNA correlated positively with the age of onset of disease.<sup>9,10</sup> To explain these imperfect correlations, additional genetic factors involving the mtDNA or the nuclear genome have been hypothesised to modulate the expression of the primary pathogenic mutations of mtDNA.<sup>9</sup> Nucleotide sequence analysis of the proband's muscle mtDNA did not show any additional nucleotide change of the tRNA<sup>Lys</sup> and tRNA<sup>Leu(UUR)</sup>, which represent two genetic hot spots in maternally inherited syndromes.<sup>21</sup> We also excluded the T9957C mutation at the gene for subunit III of cytochrome c oxidase which has been recently reported in MELAS.<sup>22</sup>

In conclusion, the presented pedigree challenges the known correlations between the clinical phenotype and the genotype of the mtDNA related disorders, and it indicates that a pure MERRF syndrome must be actually considered as a possible phenotype of the A3243G mutation at the tRNA<sup>Leu(UUR)</sup>. Further molecular genetic studies are needed to disclose other alterations of the mtDNA which could account for the clinical oddity of the described pedigree.

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