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Short communication

MERRF: Clinical features, muscle biopsy and molecular genetics in Brazilian patients

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ABSTRACT

Myoclonic epilepsy with ragged red fibers (MERRF) is a mitochondrial disease that is characterized by myoclonic epilepsy with ragged red fibers (RRF) in muscle biopsies. The aim of this study was to analyze Brazilian patients with MERRF. Six patients with MERRF were studied and correlations between clinical findings, laboratory data, electrophysiology, histology and molecular features were examined. We found that blood lactate was increased in four patients. Electroencephalogram studies revealed generalized epileptiform discharges in five patients and generalized photoparoxysmal responses during intermittent photic stimulation in two patients. Muscle biopsies showed RRF in all patients using modified Gomori-trichrome and succinate dehydrogenase stains. Cytochrome c oxidase (COX) stain analysis indicated deficient activity in five patients and subsarcolemmal accumulation in one patient. Molecular analysis of the $tRNA^{Lys}$ gene with PCR/RFLP and direct sequencing showed the A8344G mutation of mtDNA in five patients. The presence of RRFs and COX deficiencies in muscle biopsies often confirmed the MERRF diagnosis. We conclude that molecular analysis of the $tRNA^{Lys}$ gene is an important criterion to help confirm the MERRF diagnosis. Furthermore, based on the findings of this study, we suggest a revision of the main characteristics of this disease.

1. Introduction

Myoclonic epilepsy with ragged red fibers (MERRF) is a mitochondrial disorder that includes myoclonus, epilepsy, ataxia, and ragged-red fibers (RRF) in muscle biopsies (DiMauro et al., 2002; Fukuhara et al., 1980). Significant phenotypic heterogeneities are frequently observed in maternal lineage family members of MERRF pedigrees (Arruda et al., 1990; DiMauro et al., 2002; Shoffner and Wallace, 1992; Zeviani and Di Donato, 2004). Less frequently observed features of MERRF can include dementia, cardiomyopathy, lipomatosis, neuropathy, and optic atrophy (Fukuhara et al., 1980).

After clinical and histological description was established, a point mutation in the mitochondrial DNA (mtDNA) was found to be associated with MERRF. Shoffner and Wallace (1992) described a different point mutation that affected a transfer RNA (tRNA) gene in the mtDNA of MERRF patients.

To the best of our knowledge, only one patient in Brazil has been identified with this disease since its initial identification (Arruda et al., 1990). In this study, we analyzed clinical and laboratory manifestations, brain images, electroencephalogram (EEG) patterns, histology and molecular findings among Brazilian patients with MERRF.

2. Materials and methods

A retrospective analysis of 1282 muscle biopsies performed from January 2001 to January 2010 revealed six patients with MERRF based on the following clinical features: 1) myoclonus, 2) generalized epilepsy, and 3) RRF. Patient consents for muscle biopsies and mtDNA tests were obtained in an outpatient clinic or during hospital admission for diagnostic investigation.

2.1. Clinical evaluation

The time of progression of MERRF was defined as the interval between the onset of epilepsy or myoclonus and the time at which a definitive diagnosis using muscle biopsies was made. Other relevant data were collected, including clinical evaluation, age, gender, medical records of other affected relatives, and antiepileptic management. The antiepileptic treatment response was classified as present, absent, or partial based on the objective improvement of myoclonus and epilepsy over the course of the drug treatment.

2.2. Laboratory analysis

Abnormal levels of blood lactate and creatine kinase (CK) in patient serum samples were measured as a proportion to reflect their increase above normal limits. Cerebrospinal fluid (CSF) proteins were classified as normal or increased.

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2.3. Brain images

Radiologic findings (i.e., computed tomographic (CT) scans or magnetic resonance imaging (MRI)) were described.

2.4. Electrophysiological findings

Interictal EEG findings were used to assess background activity (normal or slow), presence (absent or present), type (spike, sharp wave, polyspike or spike wave) and localization (focal or generalized) of epileptiform abnormalities and intermittent photic stimulation response (present or absent). The EMG pattern was classified as normal, myopathic, neurogenic or mixed (myopathic with neurogenic findings). Motor and sensory nerve conduction studies were performed. ECG abnormalities were also reported.

2.5. Muscle biopsy analysis

Muscle biopsy samples were frozen in liquid nitrogen and cryostat sections were stained for histological examination. Routine histochemical reactions were performed using standard procedures (Werneck, 1981). We determined the frequency of deficient muscle fibers by staining for cytochrome c oxidase (COX) and the frequency of RRF using modified Gomori-trichrome (MGT) and succinate dehydrogenase (SDH) stains. We counted approximately 500–1000 muscle fibers per specimen. Blood vessels (intramuscular arteries) among specimens were identified by comparing hematoxylin-eosin, MGT and SDH stains. Blood vessel characteristics identified by SDH staining were categorized as either normal (unstained or faintly stained vessel walls) or strongly succinate dehydrogenase-reactive blood vessels (SSV).

2.6. Molecular analysis

Skeletal muscle from the quadriceps or biceps brachii was used to isolate mtDNA by applying a modified phenol/chloroform method. The $tRNA^{Lys}$ gene of mtDNA was analyzed using a combination of polymerase chain reaction with restriction fragment length polymorphism (PCR/RFLP) and direct sequencing.

The PCR/RFLP analysis was designed to detect a point mutation involving an adenine (A) to guanine (G) transition at nucleotide (nt) 8344 (A8344G). Oligonucleotide primers were designed that corresponded to nt 8273–8305 (forward) and nt 8372–8345 (backward). These primers were used to amplify the putative mtDNA mutation using conventional PCR with *Taq* DNA polymerase. The backward primer contained a mismatch at position 8347 (T>G) that created an additional restriction site in amplified fragments of mutant but not wild-type mtDNA.

PCR conditions were as follows: 30 cycles of denaturation (94 °C for 1 min), annealing (55 °C for 1 min) and primer extension (72 °C for 1 min). The PCR reaction produced a 99-base pair (bp) fragment that was digested with the restriction endonuclease *Ban*II for 16 h at 37 °C.

Products from the PCR/RFLP reaction were analyzed on a 12% nondenaturing polyacrylamide gel (run at 200 V for 2 h) and bands were visualized with standard silver staining (Cunha et al., 2006). Two bands (78 and 21 bps) were observed with wild-type mtDNA, whereas the presence of the A8344G mutation created an additional *BanII* restriction site. This additional restriction site resulted in the cleavage of the 78-bp fragment into two fragments of 52 and 26 bps.

The $tRNA^{Lys}$ gene was subjected to direct sequencing if the mutation was not observed.

For the direct sequencing, conventional PCR with *Taq* DNA polymerase was used to amplify the $tRNA^{Lys}$ gene with oligonucleotide primers at nt 8273–8305 and nt 9950–9931. Initial PCR parameters consisted of 30 cycles of denaturation (94 °C for 1 min), annealing (55 °C for 1 min) and primer extension (72 °C for 1 min). This regimen was followed by purification of the PCR fragment by enzymatic methods. These purified PCR fragments were subjected to a second round of PCR using Big Dye Mix (Applied Biosystems). The secondary PCR steps consisted of 35 cycles of denaturation (96 °C for 15 s), annealing (50 °C for 15 s) and primer extension (60 °C for 4 min). This secondary regimen was followed by PCR fragment purification using the isopropanolol method. These amplified fragments were directly sequenced in the forward direction using an ABI PRISM 3100 Avant Genetic Analyzer. Sequences were compared with the revised Cambridge reference sequence (Anderson et al., 1981).

When mutations were not observed, genetic analysis was performed with PCR/RFLP of the $tRNA^{Leu(UUR)}$ gene. The presence of the A3243G mutation in the $tRNA^{Leu(UUR)}$ gene was studied using PCR/RFLP and standard procedures (Lorenzoni et al., 2009).

3. Results

The study consists of six patients (two females and four males) that were 27–54 years of age. Early development was normal in all patients. The time of disease progression ranged from 2 to 15 years with a mean time of 8.5 years. All patients studied had family members that may had been affected by mitochondrial disorders, and three of the patients (cases 2, 4 and 6) were from the same family (Table 1).

All patients had myoclonus and generalized tonic–clonic seizures starting a mean age of 28.8 years (15–40 years). Generalized tonic–clonic seizures were rare, but myoclonic seizures were frequent and only partially controlled with valproate or clonazepam. Other clinical features that were reported included cerebellar ataxia (5/6), weakness (5/6), peripheral neuropathy (3/6), multiple lipomas (2/6), headache (2/6), vomiting (2/6), dementia (2/6), ocular ptosis (2/6), ophthalmoparesis (2/6) and exercise intolerance (1/6). Hearing loss, optic nerve atrophy, short stature, cardiomyopathy and stroke-like episodes were not observed (Table 1).

Blood lactate was one to five times above normal levels in five patients (Table 1). Serum CK levels varied from normal in one patient to three times above normal limits in five patients (Table 1). CSF protein levels were elevated in only one of five patients examined (Table 1).

Table 1
Clinical findings and laboratory abnormalities in MERRF patients.

Case	1	2	3	4	5	6
Gender	M	M	F	M	M	F
Age (years)	27	34	54	51	50	43
Evolution time (years)	15	11	3	2	15	5
Family history[a]	+	+	+	+	+	+
Normal early development	+	+	+	+	+	+
Clinical manifestations						
Myoclonus	+	+	+	+	+	+
Epilepsy	+	+	+	+	+	+
Cerebellar ataxia	−	+	+	+	+	+
Weakness	−	+	+	+	+	+
Peripheral neuropathy	−	−	+	+	+	−
Multiple lipomas	−	−	+	+	−	−
Headache	+	−	−	−	−	+
Vomiting	+	−	+	−	−	−
Dementia	−	−	−	+	+	−
Ocular ptosis	+	−	+	−	−	−
Ophthalmoparesis	+	−	+	−	−	−
Exercise intolerance	−	−	−	−	−	+
Laboratory findings						
Increased blood lactate	+	+	+	−	+	+
Increased CK	−	+	+	+	+	+
Increased CSF protein	−	−	ND	−	+	−

F: female; M: male; CSF: cerebrospinal fluid; CK: creatine kinase; CSF: cerebrospinal fluid; +: presented; −: absent; and ND: not done.
[a] Same family in cases 2, 4 and 6.