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ORIGINAL RESEARCH

The prevalence of mitochondrial DNA mutations in Leigh syndrome in a Brazilian series

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OBJECTIVE: To determine the prevalence of mitochondrial DNA (mtDNA) mutations in cases with findings compatible with the diagnosis of Leigh syndrome in a Brazilian Neurological Service, and to compare those findings between the patients presenting or not these mutations.

METHOD: We analyzed six mtDNA point mutations (T8993G, T8993C, T8851C, G1644T, T9176C, and T3308C) by PCR and endonuclease digestion in 32 patients with presumptive diagnosis of Leigh syndrome, according to distribution across different age ranges.

RESULTS: We found two patients, in the subgroup under 4 years of age, presenting T8993G and T8993C mutations. Their clinical symptoms and neuroimaging findings were similar when compared to those patients not harboring these mutations.

CONCLUSION: As the molecular confirmation is pivotal for both the precise genetic counselling and therapeutic guidance, we emphasise the benefit of screening for mtDNA mutation in Leigh syndrome patients under 4 years old. Mitochondrial whole genome and whole exome analysis by next-generation sequencing technology maybe a future alternative for molecular diagnosis of this extensive genetic heterogeneous syndrome.

KEYWORDS: Leigh's syndrome; T8993G; T8993C; maternal inheritance; earlyinfantile.

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INTRODUCTION

Subacute Necrotizing Encephalopathy or Leigh Syndrome (LS) is a progressive neurodegenerative disease frequently associated with mitochondrial abnormalities. The prevalence of LS has been estimated to be 1 in 40,000[1] and is characterized by the presence of developmental delay and lactic acidosis. LS was first described in 1951,[2] when its distribution and histological aspects were reported, as presenting central nervous lesions resembling those of Wernicke's disease (athiaminosis), except that the lesions in LS tended to be more extensive, involving the striatum and sparing the mammillary bodies. The pathologic changes are usually bilateral and symmetrical, corresponding to foci of spongy necrosis with myelin degeneration, vascular proliferation and gliosis in thalami, midbrain, pons, medulla and spinal cord, as well as changes to basal ganglia, which are characteristically, but not invariably, affected.[3] The MRI findings correspond generally to the neuropathologic features, and are diagnostic criteria for the disease in children with mental or motor involution. LS is a familial or sporadic disease with a wide variety of clinical manifestations. Many authors have reported onset of symptoms in the first year of life in more than half of cases, mostly before the sixth month. However, cases with late-onset showing even greater heterogeneity in clinical presentation are also well documented.[4]

Although LS is relatively rare, the identification of this maternal type of inheritance is very important, calling for specific genetic counseling and therapeutic approach.

The aim of this study is to present the prevalence of mitochondrial DNA (mtDNA) mutations in cases with clinical symptoms and neuroimaging findings compatible with the diagnosis of LS in a Brazilian Neurological Service, and to compare those findings between the patients presenting or not these DNA mutations.

SUBJECTS AND METHODS

Patients

Thirty-two cases (22 males, 10 females) were included in the present study, and they were referred from Neuropediatrics, the Neuromuscular Disease Group of Clinical Neurology of the Hospital das Clínicas School of Medicine, the Institute of Children of the University of São Paulo and from other private and state hospitals. Four cases out of thirty-two were submitted to postmortem examination at the

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Although the diagnosis of LS is based on neuropathological findings, it is now common to make a presumptive diagnosis on the basis of characteristic clinical features, radiological abnormalities and lactic acidosis (in blood, in cerebral spine fluid or both). Thus, the inclusion criteria of our study were: 1) progressive neurologic disease with motor and intellectual developmental delay; 2) signs and symptoms of brain stem and/or basal ganglia dysfunction; 3) characteristic features of LS on neuroimaging (i.e., symmetrical lesions in the basal ganglia and/or brain stem) or typical neuropathologic changes at postmortem examination. Patients were classified in five subgroups according to the criteria proposed by van Erven,[5] based on age of onset of clinical symptoms: neonatal (up to 4 weeks of age), early infantile (1mo to 1y), infantile (>1y to 4y), juvenile (>4y to 16y), and adolescent/young adult (>16y).

Lactate levels

For lactate measurement, blood was collected in sodium fluoride tubes, at rest and after exercise whenever feasible, and cerebral spine fluid, which was kept refrigerated until immediate analysis. Lactate was measured with a Beckman Coulter analyzer by an end-point enzymatic reaction. Blood samples were obtained from 22 patients at rest, from 16 patients after exercise, and cerebral spinal fluid samples were obtained from 17 patients.

Neuroimaging studies

All patients were submitted to CT-scan and/or MRI exams (19 to CT-scan, 27 to MRI, and 14 both to CT-scan and MRI).

CT-scan were obtained in transversal images, and MRI on 1.5-T system on transversal and coronal imaging in T2-weighted spin-echo (2400/120/2 [TR/TE/excitations]), T1-weighted spin-echo (400/15/2 [TR/TE/excitations]), and FLAIR sequence (8000/160/2300 [TR/TE/inversion recovery]). Two observers (SKNM, SR), blinded to each other and to the original diagnosis, reviewed all neuroimaging data retrospectively.

Mitochondrial DNA mutation analysis by PCR and endonuclease digestion

Six mtDNA mutation points (T8993G, T8993C, T8851C, G1644T, T9176C, and T3308C) associated with LS were studied in all patients by PCR amplification of genomic DNA extracted from blood samples, and by specific endonuclease digestion of each PCR product. The following primers were used for PCR amplification: T8993GF-ccgactaatcaccacccaac; T8993GR-tgtcgtgcaggtagaggctt; T8993CF-ccgactaatcaccacc-caac; T8993CR-atgttagcggttaggcgtac; T8851F-tacccgccgcag-tactgatca; T8551R-ctataatcactgtgcccgcta; G1644TF-gtcgaa ggtggatttagcag; G1644TR-cggtcaagttaagttgagat; T9176CF-gg ccacctactcatgcacctaa; T9176CR-tgttgtcgtgcaggtagaggcttcct; T3308CF-ggtttgttaagatggcagagcccggt; T3308CR-tacaatgag-gagtaggaggttggccaccggt.

RESULTS

Patients and clinical symptoms

Clinical picture of the 32 patients. According to the classification proposed by van Erven,[5] based on age of onset of clinical symptoms as neonatal (up to 4 weeks of age), early infantile (1mo to 1y), infantile (>1y to 4y), juvenile (>4y to 16), and adolescent/young adult (>16y), the distribution of our cases was: only one neonatal, seven early infantile, seven infantile, thirteen juvenile, and four adolescent/young adult. Pyramidal signs (75%) were the most frequent clinical sign, followed by dystonia (56%), ocular movement abnormalities (43%), swallowing difficulties (41%), cerebellar signs (37%), seizures and mental developmental delay (34%), respiratory dysfunction (25%), vomiting and muscle weakness (22%) (Table 1). Interestingly, pyramidal signs were predominant after 4 years of age, observed in 16 out of 17 cases (94%); similarly, ocular movement abnormalities, cerebellar signs, swallowing and mental developmental delay were more frequent among those older than 4 years (47% and 41%).

In contrast, respiratory dysfunction and muscle weakness (33%) were more frequent among patients younger than 4 years old, mostly observed in the first year of age.

Lactate levels

Increased serum lactate levels at rest were observed in 19 out of 22 available samples, which were confirmed after exercise in 13 samples out of 16, and also in cerebrospinal fluid in 15 out of 17 samples available. Normal levels were detected in 3 patients, either at rest or after exercise, and also in cerebrospinal fluid of two patients. One of those patients with normal lactate harbored the mtDNA mutation (case b), corroborating that lactate acidosis is not always present in LS, as previously reported.[5,6] Therefore, the lactate level was not included among the parameters for presumptive diagnosis of LS in the present study.

Neuroimaging findings

MRI detected lesions mostly in putamen, caudate nucleus, periaqueductal grey matter, white matter, substantia nigra, and globus pallidum, in decreasing order of frequency, as shown in Fig. 1. There was no particular

Table 1 - Clinical signs and symptoms according to the age subgroups

	Cases		Clinical signs and symptoms									
Age subgroups	No.	(%)	Pyramidal signs	Dystonia	Ocular movement	Deglutition dysfunction	Ataxia	Seizures	Mental retardation	Respiratory dysfunction	Muscle weakness	Vomiting
Neonatal	1	3	–	1	–	–	–	–	1	1	1	–
1mo-1y	7[a]	22	4	3	2[a]	3[a]	3	4[a]	2	3[a]	4[a]	1
>1y-4y	7[b]	22	4[b]	3[b]	4[b]	3[b]	2	1[b]	1	1[b]	–	2
>4y-16y	13	40	12	9	5	5	6	5	7	2	2	2
>16y	4	13	4	2	3	2	1	1	–	1	–	2
Total	32	100	24	18	14	13	12	11	11	8	7	7

[a]indicates patient with T8993G mtDNA mutation; [b]indicates the patients with T8993C mutation.

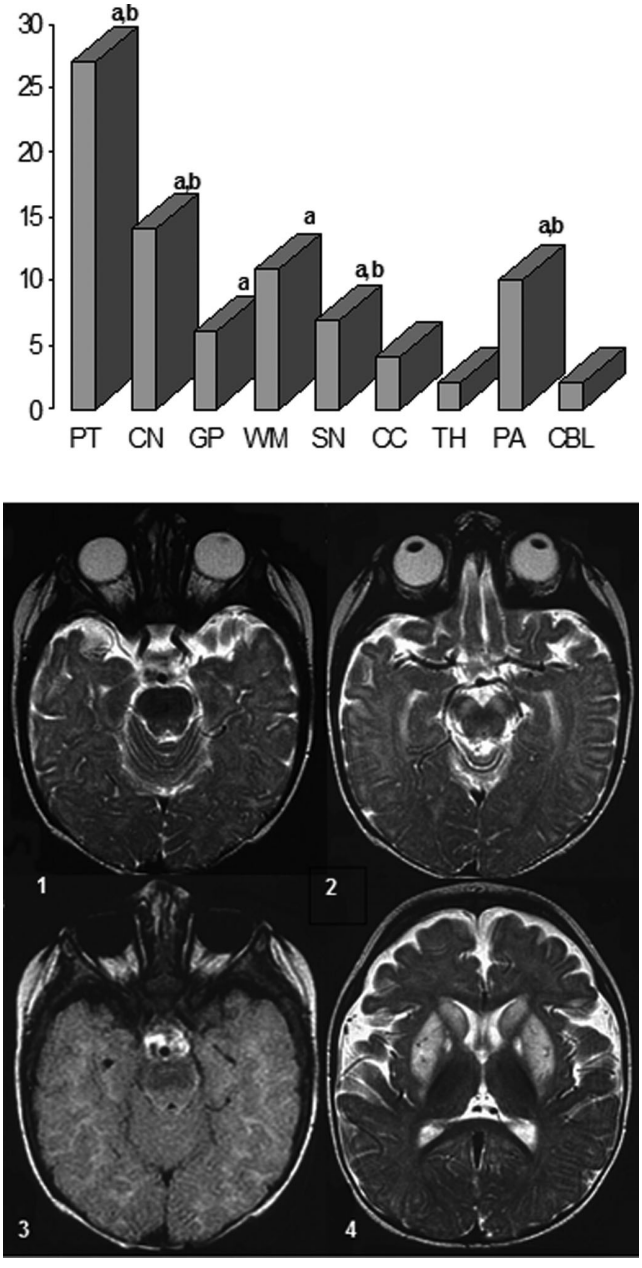

Figure 1 - Upper panel, the main involved areas in MRI. PT: putamen, CN: caudate nucleus, GP: globopallidum, WM: white matter, SN: substantia nigra, CC: corpus callosum, TH: thalamus, PA: periaqueductal grey matter, CBL: cerebellum. **a**: patient with T8993G mutation, **b**: patient with T8993C mutation. Lower panel, serial axial MRI of patient **a**. **1, 2 and 4:** T2-weighted images (2400/120) showing hypersignal on midbrain tegmentum, substantia nigra, putamen, head of caudate nucleus and globopallidum, with bilateral and symmetrical distribution. **3**: FLAIR sequence (8000/160/2300) better detecting the lesion on midbrain tegmentum.

pattern of the distribution of lesions for patients with mtDNA mutation.

mtDNA analysis

Two patients presented mtDNA mutations: T8993G and T8993C (Fig. 2), and their clinical pictures overlapped with those without mutation (Table 1 and Fig. 1).

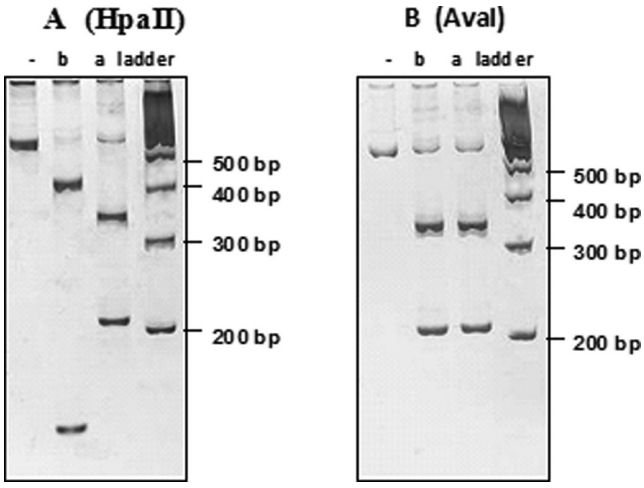

Figure 2 - The polyacrylamide gel electrophoresis of PCR products digested by HpaII (A) and AvaI (B) endonucleases. HpaII identifies only the T8993G mutation, whereas AvaI identifies both mutations. Note that patient **a** presents the T8993G mutation, in which HpaII cut the 551bp PCR product into two fragments of 347 and 208 bps. Patient **b** presents T8993C mutation, showing the same fragments when cut with AvaI, but not with HpaII. Both patients present a heteroplasmic condition, with high proportion of mtDNA mutant. Patient **b** presents, in addition, a non-pathologic polymorphism at position A8784G, confirmed by automatic sequencing, which was first detected by the different pattern of migration when cut with HpaII.

DISCUSSION

This study included 32 patients with clinical symptoms, and neuroimaging findings compatible to LS. The affected brain areas as shown by CT-scanning and MRI were similar, only with a greater number of lesions detected by the MRI, as expected. The MR-FLAIR sequence was more sensitive in demonstrating brainstem involvement than the classical T2-weighted sequence.

There are three different modes of inheritance in LS: X-linked recessive, autosomal recessive, and maternal due to mtDNA mutations.[7] Holt et al, in 1990,[8] were the first to demonstrate the T8993G mutation in mtDNA, being the most frequently occurring in LS.[9] This mutation results in the substitution of leucine, a hydrophilic amino acid by arginine, a hydrophobic amino acid. Arginine is positively charged and blocks proton translocation while impairing the ATP synthesis. A low quantity of this mutation leads to a condition known as NARP, characterized by: peripheral neuropathy, ataxia, retinitis pigmentosa, seizures, and dementia. One of our patients presented this mutation exceeding a 90% level, in proportion to the wild mtDNA. A second patient presented a T8993C point mutation, the second most frequent mtDNA mutation associated with LS, as described by De Vries et al.[10] Apparently, this mutation is related to a less severe phenotype. We detected only two out of 32 cases with mtDNA mutation (6%) with clinical and neuroimaging criteria used for the diagnosis of LS. However, when we analyze the same casuistry distributed across different age ranges, we found 13% positivity in the subgroup under 4 years of age (2 out of 15). A thorough phenotype analysis of these 2 patients with mtDNA mutation did not disclose any clinical clue for the presence of mtDNA alteration.

Although four other mtDNA mutations described in LS were also screened in the present casuistry, several other mtDNA mutations have been recently associated with LS.[11,12,13,14] This stresses the necessity for a more thorough search for mitochondrial genome mutations. To this end, large scale sequencing technologies would be helpful to detect these cases. However, LS may also be a feature of deficiency of any of the mitochondrial respiratory chain complexes: complex I to V, encoded by nuclear genes (OMIM number: 252010, 252011, 124000, 220110, 604273); of components of the pyruvate dehydrogenase complex (OMIN 238331, 300502, 308930); of deficiency of coenzyme Q10;[15] and also of COX deficiency associated with the leucine-rich PPR motif-containing protein (*LRPPRC*) gene mutation.[16] Therefore, in addition to mitochondrial genome screening, whole exome sequencing analysis would be necessary to identify the molecular defect to provide a reliable genetic counseling and for future therapeutic strategies. Next generation sequencing technology offers flexible and scalable platforms for sequencing several cases at the same time, allowing in-depth coverage to detect mutations and polymorphisms from a set of known genes, up to the whole exome or genome. The procedure can be completed in a few days with a cost per nucleotide much lower than any previous automated sequencing method.[17,18] Mostly, the requirement of pico to nanograms of starting DNA/RNA amounts is another crucial advantage when dealing with limited biological material. The cost/effective ratio is decreasing over time and precise molecular diagnosis will be feasible even for an extensive genetic heterogeneous syndrome, such as LS. A continuous collection of systematic clinical, laboratorial, and neuroimaging findings such as those presented here, in spite of any drawbacks of a retrospective study, might be helpful for the establishment of an effective algorithm for such a molecular diagnosis.

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■ RESUMO

OBJETIVO: Determinar a prevalência de mutações no DNA mitocondrial (DNAmt) em casos com achados compatíveis com o diagnóstico de síndrome de Leigh em um Serviço de Neurologia brasileiro, e comparar essas descobertas entre os pacientes que apresentam ou não essas mutações.

MÉTODO: Seis pontos de mutações do DNAmt (T8993G, T8993C, T8851C, G1644T, T9176C e T3308C) foram analisados por PCR e digestão com endonuclease em 32 pacientes com diagnóstico presuntivo de síndrome de Leigh, de acordo com a distribuição em diferentes faixas etárias.

RESULTADOS: Dois pacientes no subgrupo abaixo de 4 anos de idade apresentaram as mutações T8993G e T8993C do DNAmt. Os sintomas clínicos e os achados de neuroimagens destes dois pacientes foram similares aos dos casos sem mutações detectadas.

CONCLUSÃO: Como a confirmação molecular é fundamental tanto para o aconselhamento genético como para a orientação terapêutica, enfatizamos o benefício da pesquisa de mutações no DNAmt em pacientes com fenótipo de Síndrome de Liegh abaixo de 4 anos de idade. O sequenciamento em larga escala do genoma mitocondrial e do exoma completo por tecnologia de sequenciamento de nova geração poderá ser uma alternativa futura no estabelecimento do diagnóstico molecular nesta síndrome genética extensamente heterogênea.

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