Bilateral Striatal Necrosis Associated with a Novel Mutation in the Mitochondrial ND6 Gene

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We report the molecular findings in two independent patients presenting with progressive generalized dystonia and bilateral striatal necrosis in whom we have identified a mutation (T14487C) in the mitochondrial ND6 gene. The mutation is heteroplasmic in all samples analyzed, and it fulfills all accepted criteria of pathogenicity. Transmitochondrial cell lines harboring 100% mutant mitochondrial DNA showed a marked decrease in the activity of complex I of the respiratory chain supporting the pathogenic role of T14487C.

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Mitochondrial DNA (mtDNA) point mutations have been associated with a wide range of clinical presentations ranging from pure myopathies to multisystemic disorders.[1–3] Here, we present molecular and biochemical data in two independent patients with bilateral striatal necrosis (BSN) and dystonia in whom we have identified a novel heteroplasmic mutation in the ND6 gene of mtDNA. The mutation, a T-to-C transition at nucleotide position 14487 (T14487C), leads to the substitution of methionine at amino acid position 63 by valine (M63V) and fulfills all accepted criteria of pathogenicity. Moreover, in vitro studies using a transmitochondrial cell line harboring the T14487C mutation showed a marked defect of complex I activity, suggesting that this is the disease-causing mutation.

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Patients and Methods

Patient 1

Patient 1 was a 15-year-old male adolescent who had had a normal delivery and development until age 4 years when he was admitted to our hospital because of subacute onset of gait disturbance after an episode of pneumonia. Neurological examination evidenced generalized hypotonia with left Achilles clonus and Babinski sign. Dystonic-spastic gait when walking was noticed. Brain magnetic resonance imaging (MRI) showed the presence of BSN, and there was no increase in lactic acid levels in plasma or cerebrospinal fluid. Since the initial episode, the patient has been followed up, and his disease has progressed towards a generalized asymmetric dystonia. New basal ganglia necrotic lesions in a follow-up brain MRI performed at age 13 years were observed. A muscle biopsy at age 14 years showed scattered muscle fibers positive for oxidative activity only in their periphery, suggesting a respiratory chain disorder. Currently, the patient presents with severe dysarthria and moderate mental retardation with generalized dystonia. Family history was negative for neurological disorders.

Patient 2

Patient 2 was a 15-year-old male adolescent born in a normal delivery and has healthy nonconsanguineous parents. His neurological symptoms began at age 6 years when his parents noticed external rotation of the left foot while walking. At age 9 years, upper left limb dystonia was detected without a clear-cut precipitating event. At age 10 years, a brain MRI showed the presence of BSN. The phenotype has progressed, and currently the patient is severely disabled by dystonic tetraplegia with relatively well-preserved mental functions. As in the previous case, family history was negative for neurological disorders.

In both cases, there were no abnormalities in white or red blood cells or plasma copper or ceruloplasmin levels. Both patients come from different geographical areas of Spain, and there is no evidence of a genetic relationship between them.

Molecular Genetic Studies

Muscle DNA from Patient 1 was extracted according to conventional methods, and 16 polymerase chain reaction (PCR) products overlapping the entire coding region for the seven NADH-dehydrogenase subunits and the two ATPase subunits were obtained using primers previously reported for sequencing the entire mitochondrial genome.[4] Direct sequencing of PCR products was performed using the same set of primers (forward and reverse) in an automatic sequencer (310 Automatic Sequencer; Perkin Elmer, Foster City, CA). Restriction fragment length polymorphism (RFLP) analysis adding [α-^{32}P]-dCTP in the last cycle was performed as described (hot-PCR method).[5] In brief, a 209bp fragment was amplified using the following primers: a 22-mer forward (5′-CAGCTTCCTACACTATTAAATG-3′) and a double mismatched 24-mer reverse (5′-GTTTTTTTAATTTATTT-AG**CT**GGA-3′). Because the mismatched primer creates a restriction site for *Gsu*I, this enzyme was used to quantify the proportion of mutated genomes.

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Biochemical Studies of a T14487C Transmitochondrial Cell Line

Transmitochondrial cell lines were obtained by fusion of platelets, which were isolated from the blood of Patient 1, containing 67% T14487C mutant mtDNA, with human osteosarcoma 143B cells lacking mtDNA (ρ0 cells), as described.[6] In brief, platelets were isolated from plasma by centrifugation and fused with ρ0 cells by using polyethylene glycol. After selection in uridine-lacking medium, the surviving colonies were trypsinized in a cloning ring and expanded to obtain single clones. DNA was extracted from each clone, and mtDNA was analyzed as described above.

Rotenone-dependent oxygen consumption was measured by polarography in clones harboring 100% of mutated genomes as well as in control transmitochondrial cell lines, in the presence of 5mM glutamate and 5mM malate, followed by the addition of 100μM rotenone. In addition, respiratory chain enzymes activities were measured in muscle from Patient 1 by conventional methods.

Results and Discussion

In addition to previously identified polymorphisms of the mitochondrial genome, sequence analysis of the PCR products of muscle DNA from Patient 1 showed the presence of an heteroplasmic T-to-C transition at nucleotide position 14487 in the ND6 gene (Fig 1A), which caused the substitution of methionine-63 (AUG) with a valine (GUG) (ND6 is coded in the L-strand). RFLP analysis showed that the T14487C mutation was heteroplasmic with a high proportion of mutant mtDNA in muscle (93%) and in blood (67%; see Fig 1B). Interestingly, two blood samples taken 6 years apart showed a significant decrease of mutational load in peripheral blood cells (from 77% in 1995 to 67% in 2001). This is not surprising, because the same phenomenon, a time-dependent decrease in the mutational load in peripheral blood cells, has been shown for the A3243G mutation, which has been attributed to a replicative advantage of wild-type cells in tissues with mitotic capacity.[7] Moreover, the presence of the mutation in the blood of the patient's mother (26% of mutated genomes) suggests that the patient inherited the mutation from his mother who probably did not show the phenotype because the mutation load could

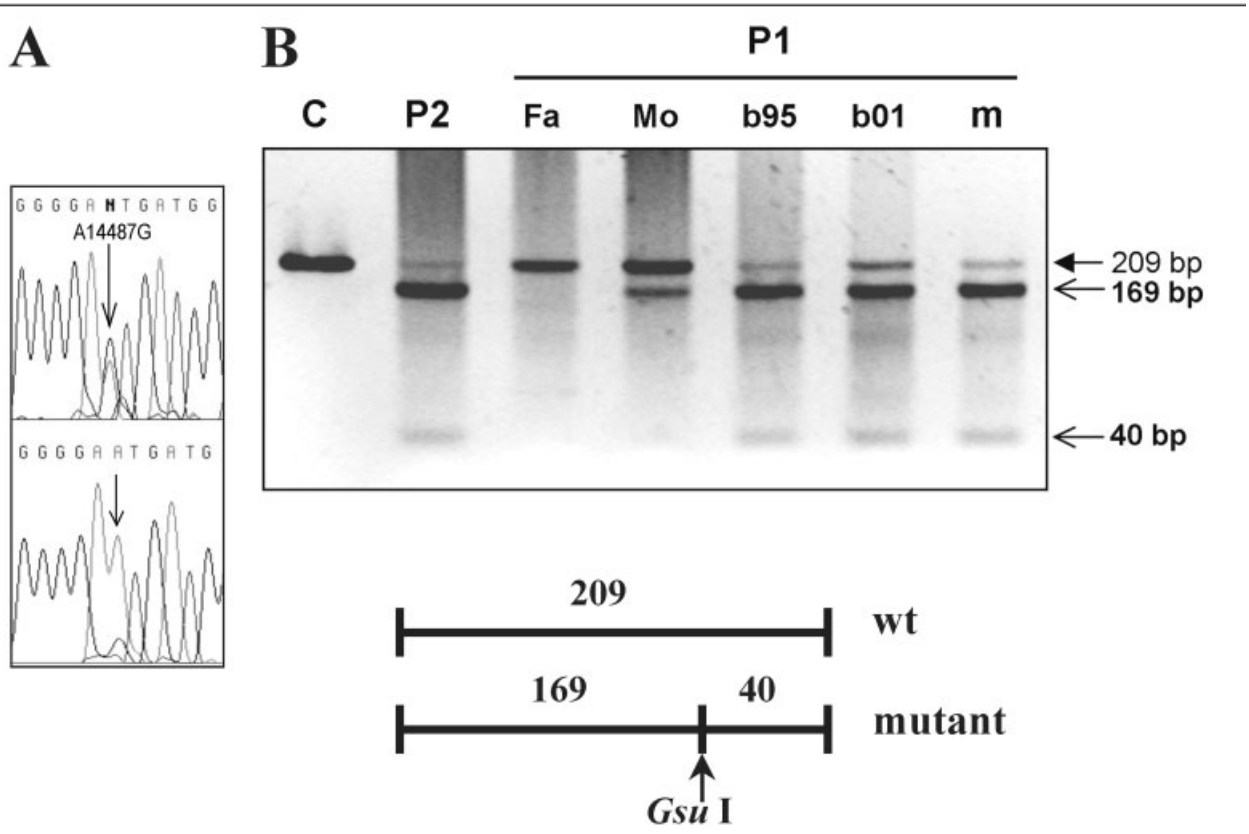

Fig 1. Molecular analysis of the T14487C mutation. (A) Automated sequence analysis of the ND6 gene (H-strand) showing the heteroplasmic change (A-to-G) at nucleotide position 14487; top sequence corresponds to Patient 1 (muscle) and the bottom sequence to the patient's mother (blood) (with majority of wild-type mitochondrial DNA). (B) RFLP analysis of the T14487C mutation. In the presence of the mutation, the polymerase chain reaction product (209bp) is digested into two fragments of 169 and 40bp (recognition by Gsu*I). C = normal control; P2 = Patient 2 (blood); Fa = father of Patient 1 (blood); Mo = mother of Patient 1 (blood); b95 = Patient 1 (blood from 1995); b01 = Patient 1 (blood from 2001); m = Patient 1 (muscle).*

be below a pathogenic threshold. The identification of the mutation in Patient 1 prompted us to study Patient 2 who showed a significant similar phenotype. As shown in Fig1B, this patient had a high level of mutation in blood (94%), and, although muscle DNA was not available for the analysis, we can reasonably speculate that he had a similar or higher level in muscle or brain tissue. Unfortunately, no muscle tissue or DNA from maternal relatives was available for analysis to prove that the mutation was transmitted throughout the maternal germline; therefore, we could not exclude the possibility that the mutation arose de novo. Sequencing analysis of the hypervariable regions 1 and 2 from the D-loop confirmed that both patients were not related.

Biochemical analysis of muscle from Patient 1 showed isolated complex I deficiency: complex I + III: 4.93 (reference range, 12–56); complex II: 9.33 (reference range, 10–25); complex III: 73.79 (reference range, 55–259); complex IV: 99.9 (reference range, 59–170; results expressed in nanomols per minute per milligram protein). Furthermore, rotenone-dependent oxygen consumption in transmitochondrial cell lines harboring 100% of mutated genomes showed undetectable levels (almost 0fmol O_2/cell/min; Fig 2) when compared with control transmitochondrial cells (1,758fmol O_2/cell/min). These data strongly suggest that the T14487C mutation abolishes complex I activity.

M63V is highly conserved throughout evolution, and, interestingly, it is located in the codon immediately preceding the Lieber's hereditary optic neuropathy-associated mutations C14482G, C14482A, and T14484C, whose pathogenic role has been well established.[8,9] On the other hand, the BSN and dystonia phenotype has been associated with specific alterations of complex I activity, as assessed by in vitro studies on platelets[10] and uncloned transmitochondrial cell lines from patients with idiopathic dystonia.[11] Further evidence supporting the hypotheses that ND6 mutations may have a key role in the presentation of dystonic phenotypes has been the recent identification of three patients harboring the 3460, 14459, and 14484 mutations who developed a Leigh-like neurological disorder whose landmark was the presence of bilateral brainstem lesions in the MRI.[12]

A better understanding of the genotype–phenotype relationship in mitochondrial patients can be achieved only if mutation-specific phenotypes are identified. Although most differences in the clinical presentation of these patients may be attributed to variations in the heteroplasmy levels or differences in the distribution of mutant mtDNA in different tissues, it is generally accepted that some mutations tend to present with clinically well-defined phenotypes, such as the stroke-like episodes due to the A3243G mutation or the presence

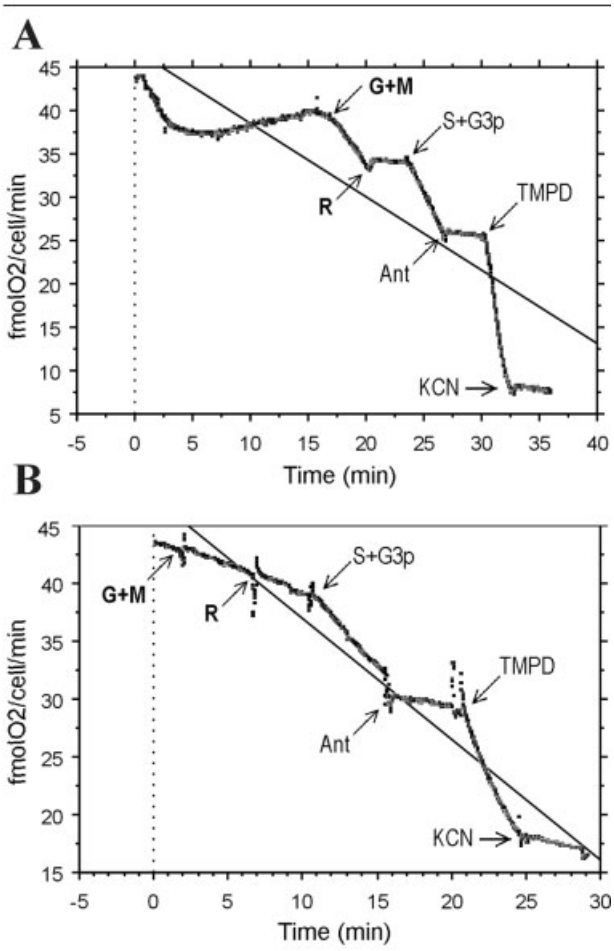

Fig 2. Oxygen consumption. (A) Control cells (143B). (B) Transmitochondrial cells homoplasmic for the T14487C mutation. G+M = glutamate + malate; R = rotenone; S+G3p = succinate + glycerol-3-phosphate; Ant = antimycinel; TMPD = N,N,N′,N′*-tetramethyl*-p-*phenylenediamine; KCN = potassium cyanide.*

of multiple lipomas, which have been found only in patients harboring mutations in the tRNALys gene. The identification of different mutations in the ND6 and ATP6 genes[13] associated with BSN and dystonia suggests a gene-specific involvement in the presentation of this genotype.

In conclusion, several lines of evidence support the pathogenic role of the T14487C mutation.

1. T14487C has been identified in two independent patients who presented with virtually the same clinical phenotype whose landmark was a neurological disorder characterized by slowly progressive generalized dystonia with BSN. In both cases, mutant mtDNA was heteroplasmic, and heteroplasmy is a common feature of pathogenic mtDNA mutations.

2. M63V is completely conserved among mammals, and it can be found down the evolution scale until invertebrates such as *Drosophila melanogaster*, although it is not conserved among lower eukaryotes and prokaryotes. This significant degree of conservation of M63 suggests that its substitution by valine may lead to impairment of complex I activity.
3. The mutation was not present in a large series of 150 individuals from the same genetic background.
4. Biochemical studies in muscle and in a transmitochondrial cell line harboring homoplasmic levels of mutant mtDNA showed a specific alteration of complex I activity establishing a link between the mutation and the biochemical phenotype.

These findings suggest that mitochondrial ND genes should be studied in patients with maternally inherited or sporadic BSN and dystonia, particularly if complex I deficiency is demonstrated.

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Voltage-Gated Potassium Channel Antibodies in Limbic Encephalitis

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We found voltage-gated potassium channel (VGKC) antibodies in 4 of 15 patients with limbic encephalitis (LE). Two patients with idiopathic LE had high VGKC antibody levels (>800pM; controls <100pM), that fell in parallel with a clinical response to immunotherapy. Two patients with lower VGKC antibodies (170pM, 300pM) had lung cancer (radiological evidence only in one) and the LE improved with immunotherapy in one. The other 11 patients without VGKC antibodies had paraneoplastic LE and eight onconeural antibodies (Hu in 6; Ma2 in 2). VGKC antibodies do not unambiguously discriminate between idiopathic or paraneoplastic LE but probably indicate a good response to immunotherapy.

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Limbic encephalitis (LE) is a paraneoplastic neurological syndrome often associated with small cell lung carcinoma (SCLC) and more rarely with testicular, breast, and other tumors (reviewed by Gultekin and colleagues[1]). LE causes short-term memory loss, seizures, confusion, and behavioral changes. The magnetic reso-

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