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Reversion of mtDNA depletion in a patient with TK2 deficiency

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Abstract—Mutations in the thymidine kinase 2 (TK2) gene cause a myopathic form of the mitochondrial DNA depletion syndrome (MDS). Here, the authors report the unusual clinical, biochemical, and molecular findings in a 14-year-old patient in whom pathogenic mutations were identified in the *TK2* gene. This report extends the phenotypic expression of primary TK2 deficiency and suggests that factors other than TK2 may modify expression of the clinical phenotype in patients with MDS syndrome.

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The identification of mutations in genes controlling the mitochondrial nucleotide pool has shed new light into the pathologic mechanisms of mitochondrial DNA (mtDNA) depletion syndromes (MDS). Mutations in thymidine kinase-2 (*TK2*) have been reported in patients with the myopathic form of MDS[1] and mutations in the deoxyguanosine kinase (*dGK*) gene in patients with the hepatocerebral form of MDS.[2] In addition, loss-of-function mutations in the thymidine phosphorylase gene have been identified in patients with mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), an autosomal-recessive disease with depletion and multiple deletions of mtDNA.[3]

In 2001, Barthélémy et al.[4] described an 8-year-old boy who had an isolated myopathic form of MDS. Concentration of mtDNA in muscle was less than 10% of normal, but activities of respiratory chain enzymes and steady-state levels of mitochondrial transcripts in muscle were normal. This discrepancy was attributed to a transcriptional compensatory mechanism. Symptoms, however, worsened over the next 6 years. We re-evaluated this patient at age 14. Here, we report additional clinical, biochemical, and morphological data, as well as evidence of TK2 deficiency.

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Patient and methods. *Case report.* This 14-year-old boy was noted to have mild neonatal hypotonia. Early development was

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normal. At age 3, he had difficulty running and fell frequently, which led to his first pediatric evaluation that revealed evidence of a myopathy.[4] A muscle biopsy showed ragged-red fibers (RRF); however, respiratory chain enzyme activities were normal. At age 8, a second muscle biopsy revealed RRF that were also cytochrome *c* oxidase (COX)–deficient.[4] Again, activities of mitochondrial respiratory enzymes were normal. Severe depletion of mtDNA in muscle was detected.[4] The child's weakness worsened, and he became wheelchair-bound at age 13.

Over the past 2 years, weakness has spread to respiratory muscles, causing dyspnea while talking. He has also developed dysphagia and has had three bouts of pneumonia. He is cognitively intact. He has overt facial weakness with ptosis and incomplete eye closure; however, extraocular movements are full. He has a high-arched palate and sluggish palatal movements. Hearing is normal. He has marked atrophy of proximal limb muscles (figure 1) with pseudohypertrophic calves. There is scapular winging, equinus deformities, and kyphoscoliosis. Weakness affects all muscles, but more so limb-girdle muscles (iliopsoas and quadriceps femoris, 2/5). He cannot sit without support due to axial weakness. The scapulohumeral muscles are the least affected (4−/5 biceps brachii and deltoids). Sensory and cerebellar functions are normal. Tendon reflexes are absent. Pulmonary function tests confirmed respiratory muscle weakness. Although he denies visual problems, visual evoked potentials showed delayed P100 responses, and an electroretinogram disclosed absence of b-wave responses. Cerebral, cardiac, renal, and hepatic functions remain normal.

Biochemistry and immunohistochemistry. Respiratory chain enzyme activities were measured in muscle extracts according to previously established methods.[5] Four μm-thick frozen sections were used for immunohistochemistry with a monoclonal anti-DNA antibody (Chemicon, Temecula, CA) using fluorescein as a fluorochrome.

DNA analysis. Southern blot analysis was performed using 5 μg of total muscle DNA by standard procedures. The membrane was hybridized with two probes (one mtDNA fragment of the 12S rRNA gene and one nuclear fragment of the 18S rRNA gene). The *TK2* gene was sequenced.[6]

TK2 activity and structure. TK2 activity was measured radiochemically using isolated mitochondria of fibroblasts from the patient and from ten controls as previously described.[7] The structure of TK2 was visualized by the programs MOLSCRIPT and RASTER 3D (Avatar Software, Stockholm, Sweden) and structural effects of the mutations were studied with the program O in a dNK-based model.[8]

Results. The muscle biopsy showed severe atrophy and heavy infiltration with connective tissue and fat. By histochemistry, approximately 10 to 20% of the residual muscle fibers were COX-negative and 7% were RRF (not shown). Activities of the respiratory chain complexes containing mtDNA-encoded subunits, normalized to citrate synthase, were all decreased (complex IV 29%, complexes II+III 15%, complex I 55%, and complexes I+III 69%).

By Southern blot analysis, no mtDNA depletion was observed in the muscle biopsy (see figure E-1A on the *Neurology* Web site). This result was confirmed by immunohistochemistry with anti-DNA antibody that revealed normal cytoplasmic staining in muscle (not shown). The presence of a single point mutation or a single micro-rearrangement in mtDNA genes was excluded by sequencing the entire mitochondrial genome using skeletal muscle DNA.

Sequence analysis of the coding region of the *TK2* gene revealed two heterozygous mutations in exon 8: a C-to-G transversion at nucleotide 462 (resulting in the substitution of arginine for glycine at codon 152, R152G) and a trinucleotide deletion abolishing lysine at codon 171 (K171del) (Genbank ID, NP_004605) (see figure E-1B on the *Neurology* Web site). Analysis of the subcloned PCR fragment of exon 8 showed that the mutations were allelic (not shown).

Biochemical studies of the isolated mitochondria from the patient's skin fibroblasts showed a severe decrease of TK2 activity (0.089 ± 0.05 nmol/minute/g protein) as compared with ten controls (9.9 ± 3.7 nmol/minute/g protein).

Discussion. Several lines of evidence support the pathogenicity of the *TK2* mutations (K171del/R152G) in this patient: (1) the genotype is consistent with the biochemical phenotype as the patient showed reduced *TK2* activity in fibroblasts; (2) the K171 and R152 residues are well conserved in all deoxynucleoside kinases as they form part of the dNK domain of these enzymes; (3) *TK2* structural analysis shows that K171del probably disrupts the substrate-binding site (see figure E-2 on the *Neurology* Web site), whereas the R152G mutation may destabilize protein folding; (4) parental DNA was not available for the study as the patient was adopted, but analysis of the subcloned exon 8 fragment showed that both mutations were allelic, implying autosomal-recessive inheritance; and (5) both mutations were not found in a series of 100 normal controls.

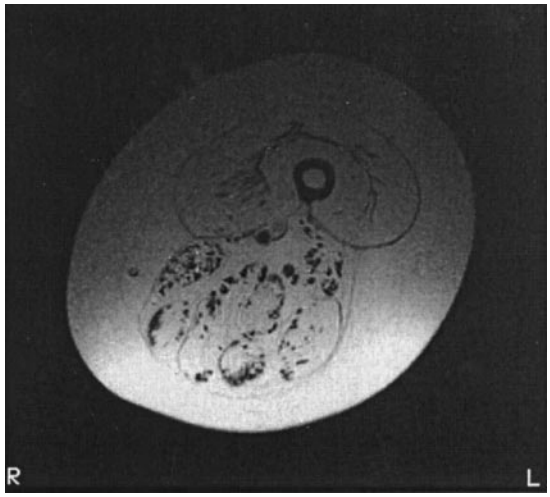

Figure 1. MRI of the patient's thigh showing profound muscle atrophy and diffuse fat infiltration.

Like previously described individuals with *TK2* mutations, our patient also has a progressive myopathy.[1] Over the past 6 years, he has developed marked muscle atrophy (see figure 1) and severe pharyngeal and respiratory muscle weakness that have led to multiple respiratory infections. In addition to muscle involvement, he has electrophysiological evidence of optic neuropathy and retinopathy. This is not surprising because two other patients with myopathy and *TK2* mutations also had CNS involvement.[6]

It is not readily apparent why some individuals harboring *TK2* mutations have isolated myopathy and others also have CNS involvement. Tissue-specific expression of mtDNA depletion may be related to differential expression of other genes regulating the mitochondrial dNTP pool, such as nucleoside diphosphate kinase, mitochondrial 5' nucleotidase, and deoxynucleotide carrier, which preferentially imports deoxynucleotide diphosphates into mitochondria and is expressed at low levels in muscle.[9] Therefore, mitochondrial importation of thymidine nucleotides in muscle may not be sufficiently effective to bypass the *TK2* defect.[1]

The most striking findings in this patient's muscle at age 14 are the normal level of mtDNA and impaired respiratory enzyme activities in contrast to the severe mtDNA depletion and normal mitochon-

drial enzyme activities in the biopsy obtained at age 8 years. A plausible explanation for these discrepancies is that the mtDNA-depleted fibers became atrophic or died while surviving non-atrophic fibers, which were able to maintain nucleotide pool homeostasis, had normal amounts of mtDNA. Interestingly, muscle atrophy has been also reported in another patient harboring a homozygous mutation in the *TK2* gene.[6] In that 3 year-old child, a muscle biopsy showed hypertrophic fibers intermixed with groups of atrophic fibers. These are, to our knowledge, the only two living patients with *TK2* deficiency, which suggests muscle atrophy developed in patients when they do not die at an early age. Paradoxically, the residual muscle fibers retaining normal mtDNA levels demonstrate respiratory chain defects as assessed by both biochemical and morphological studies. These enzyme abnormalities could be due to an accumulation of mtDNA point mutations, which is consistent with the recent identification of somatic point mutations of mtDNA in muscle and other affected tissues in patients with MNGIE, another disease due to a defect in a gene controlling the homeostasis of the mitochondrial nucleotide pool (Y. Nishigaki and M. Hirano, manuscript in preparation).

Severe and progressive muscle atrophy with evidence of mitochondrial dysfunction should alert the clinician to the possible diagnosis of TK2 deficiency. Measurement of TK2 activity in cultured fibroblast may be a reliable diagnostic test. Among reported MDS patients with *TK2* mutations,[1,9] our patient stands out for his late onset and long survival. As residual TK2 activity is similar in all patients, these clinical differences may be due to compensatory mechanisms in the nucleotide metabolism pathways.

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