Deleterious Mutation in the Mitochondrial Arginyl–Transfer RNA Synthetase Gene Is Associated with Pontocerebellar Hypoplasia

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Homozygosity mapping was performed in a consanguineous Sephardic Jewish family with three patients who presented with severe infantile encephalopathy associated with pontocerebellar hypoplasia and multiple mitochondrial respiratory-chain defects. This resulted in the identification of an intronic mutation in *RARS2,* the gene encoding mitochondrial arginine–transfer RNA (tRNA) synthetase. The mutation was associated with the production of an abnormally short *RARS2* transcript and a marked reduction of the mitochondrial $tRNA^{Arg}$ transcript in the patients' fibroblasts. We speculate that missplicing mutations in mitochondrial aminoacyl-tRNA synthethase genes preferentially affect the brain because of a tissue-specific vulnerability of the splicing machinery.

Of the 85 subunits that form the mitochondrial respiratory chain, 13 are synthesized within the mitochondria and depend on a separate mitochondrial translation system. This apparatus consists of 2 ribosomal RNA (rRNA) and 22 tRNA transcripts encoded by the mitochondrial genome and a large set of nuclear-encoded ribosomal proteins, aminoacyl-tRNA synthetases, tRNA modification enzymes, and translation factors. For the past 2 decades, >130 pathogenic mutations in the mitochondrial tRNA and rRNA genes have been registered in the Mitomap database. The clinical phenotypes range from mild, late-onset disorders, such as age-related sensorineural hearing impairment, ocular myopathy, and diabetes mellitus, to devastating and usually fatal infantile disorders, such as Leigh syndrome.

More recently, defects in a small number of nuclear-encoded components of the mitochondrial translation machinery, including three translation factors (EFG1, EF-Ts, and EF-Tu)[1–3] and a single ribosomal protein,[4] were identified. The patients presented in early infancy with encephalopathy and marked lactic acidosis accompanied by cardiomyopathy and liver failure, with mutations of EF-Ts and EFG1, respectively. Onset during childhood and lack of significant acidosis were characteristic for patients with mutations in the tRNA modification factor PUS1[5] who presented with myopathy and anemia. Slowly progressive disease was also described in patients with leukodystrophy due to mutations in the mitochondrial aspartyl-tRNA synthethase.[6]

We investigated three patients, the products of a Sephardic Jewish consanguineous marriage, who had infantile encephalopathy and a putative defect in mitochondrial translation. Patient II-2, the second child of the parents, was a girl born at 41 gestational wk by uncomplicated vaginal delivery after an uneventful pregnancy. Her Apgar scores were 8 at 5 min and 9 at 10 min, birth weight was 2,700 g, length was 51 cm, and head circumference was 34 cm. Physical examination at several hours of age revealed generalized hypotonia and poor sucking. Brain magnetic resonance imaging (MRI) at age 3 d (fig. 1*a*) disclosed cerebellar and vermian hypoplasia but normal brain volume. Recurrent apnea since age 1 wk was controlled by phenobarbital, but intractable seizures that started at age 2 mo were resistant to multidrug therapy. She failed to thrive because of poor sucking and gastroesophageal reflux. After the insertion of gastrostomy tube and funduplication, her weight gain became satisfactory, but microcephaly became evident (at age 1 year, head circumference was 40 cm). The patient did not attain any developmental milestones, and her muscle tone became spastic. The results of repeated fundoscopic examinations were normal. Serial brain MRI revealed progressive atrophy of the cerebellum, pons, cerebral cortex, and white matter (fig. 1). The patient died at age 16 mo.

Thorough investigations aimed toward elucidation of the severe neurological disease of patient II-2 performed throughout her life, including nerve and skin biopsy for inclusion bodies, did not disclose any abnormality. Specifically, levels of creatine kinase, plasma lactate, and amino acids; findings from liver and renal function tests, isoelectrofocusing of transferrins, karyotype analysis, and FISH analysis for Prader-Willi and Miller-Dieker syndromes; and acylcarnitine profile and urinary organic acid profile were all normal. Enzymatic investigations in leukocytes excluded Krabbe disease, metachromatic leukodystrophy, GM1 gangliosidosis, mannosidosis, and Tay

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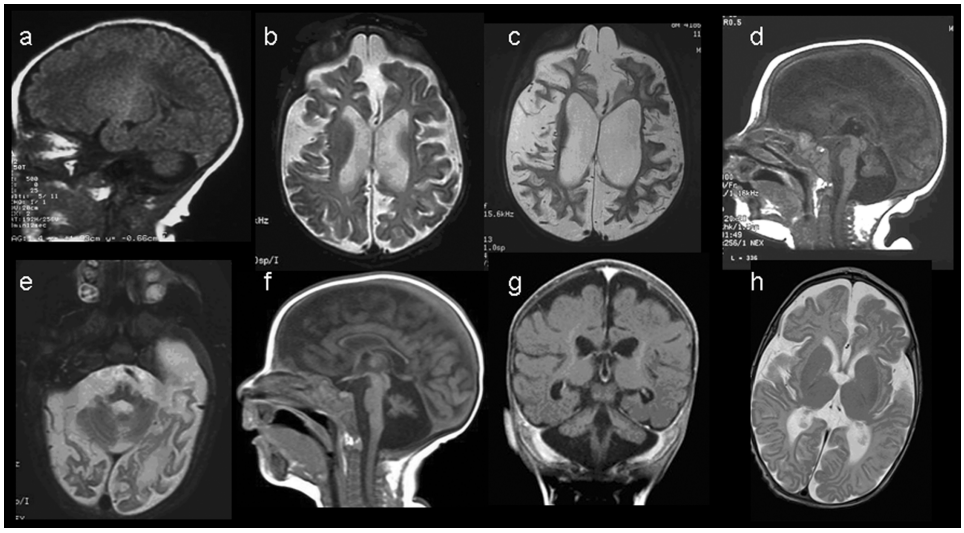

Figure 1. Brain MRI of patient II-2 (*a–e*) and patient II-5 (*f–h*). *a,* Sagittal T1-weighted image of patient II-2 obtained at age 3 d, showing hypoplasia of the cerebellum and the vermis, with progression at 5 mo (*d*). Brain volume was relatively preserved at age 3 d (*a*) but decreased at age 5 mo (*b*) and at age 9 mo (*c*), mainly because of white-matter depletion (panels b and c are axial T2-weighted images). *e,* Axial T2-weighted image of patient II-2 obtained at age 9 mo, showing severe pontine atrophy. Midsagittal (*f*) and coronal (*g*) T1-weighted images of patient II-5 obtained at age 3 mo show hypoplasia of the cerebellum and the vermis. *h,* Axial T2-weighted images disclosing some parenchymal loss, mostly of the white matter, with relative sparing of the basal ganglia and cortex.

Sachs disease. Findings from echocardiogram, electromyogram, and the nerve conduction velocity test were normal, but electroencephalography (EEG) repeatedly disclosed generalized epileptic activity with attenuation, which was more prominent over the right hemisphere. In homogenate of skeletal muscle obtained at age 5 mo, the activity of cytochrome *c* oxidase (complex IV) was 0.65 μmol/min/g protein (control $\pm$ SD 2.80 $\pm$ 0.52), that of succinate:cytochrome *c* reductase (complexes II and III) was 0.18 μmol/min/g protein (0.70 $\pm$ 0.23), that of NADH:cytochrome *c* reductase (complexes I and III) was 0.06 μmol/min/g protein (1.02 $\pm$ 0.38), that of NADH dehydrogenase (complex I) was 13.25 μmol/min/g protein (35.48 $\pm$ 7.07), and that of succinate dehydrogenase (complex II) was 0.77 μmol/min/g protein (1.00 $\pm$ 0.53) (Dr. S. Shanske, College of Physicians and Surgeons, Columbia University, New York).

Patient II-4, the fourth child in the family, was a boy who was markedly hypotonic and lethargic since birth. He was difficult to arouse and fed poorly because of inadequate sucking. Findings from brain ultrasound at age 3 d were normal, plasma lactate was 3 mM (normal <2.4 mM), and levels of creatine kinase, amino acids, very-long-chain fatty acids, and phytanic and pristanic acids and urinary organic acid profile were all normal. No developmental milestones were reported, and the patient was found dead in his crib at age 7 wk. A skin biopsy sample for fibroblast culture was obtained immediately after death. The enzymatic activities of the mitochondrial respiratory-chain complexes I–V in mitochondria isolated from the fibroblasts were determined by standard spectrophotometric methods.[7] Complex I activity normalized for citrate synthase activity was reduced to 60% of the control mean, but complex II–IV activity was within the normal range.

Patient II-5, the fifth child in the family, was a girl born at term by vaginal delivery after an uneventful pregnancy. Of note, fetal brain sonography at 20 wk gestation disclosed normal brain anatomy. Birth weight was 2,650 g, and head circumference was 32.2 cm. A thorough neurological examination on the 1st d of life produced normal findings, but a single apneic episode was noted on the 2nd d of life. At age 3 wk, poor feeding, lethargy, and generalized hypotonia became evident; results of fundoscopy and EEG were normal. Pharyngeal muscle incoordination was demonstrated by fiber-optic examination; thereafter, normal weight gain was sustained by nasogastric tube feeding. Repeated episodes of respiratory failure, initially attributed to an insufficient respiratory drive, were resolved by tracheostomy with restoration of spontaneous breathing. At age 4 mo, she experienced generalized seizures, and her head growth was arrested. Physical examination revealed a pale, ill-appearing child with limb spasticity, bilateral elbow contractures, and truncal hypotonia. She responded to auditory stimuli but had no meaningful interaction with her surroundings. Brain MRI performed at age 3 mo revealed general atrophy, and the pons and the cerebellum were the most affected (fig. 1*f*–1*h*). Plasma lactate level was normal, but cerebrospinal fluid (CSF) lactate level was 3–4 mM (normal <2 mM). There was no indication of any extracerebral involvement. Specifically, results of renal and liver function tests, levels of creatine kinase, plasma amino acids profile, carnitine and acylcar-

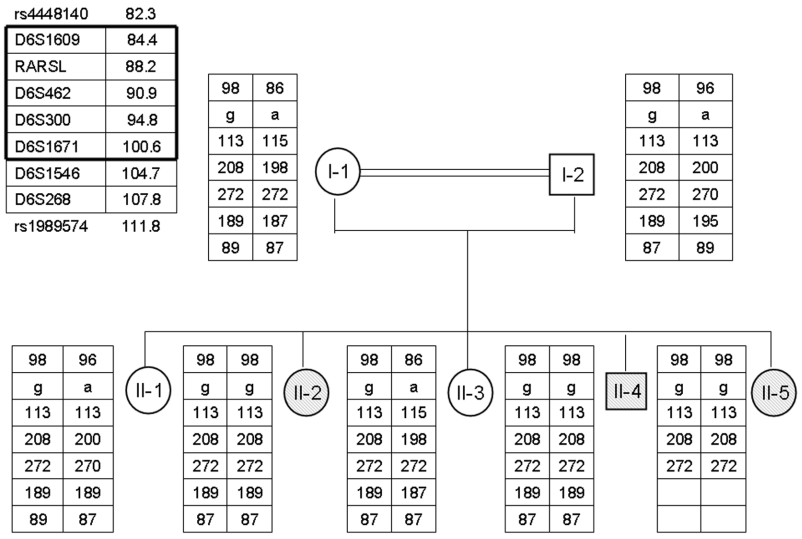

Figure 2. The family members' haplotype along the critical region on chromosome 6. The polymorphic microsatellite markers and their chromosomal locations (in Mb) are given in the upper left panel. The SNPs bordering the homozygous region are given above and below the panel. The linked region is bordered by bold lines.

nitine levels, and urinary organic acids profile were normal. In muscle mitochondria obtained at age 1 mo, the activity of complex IV was reduced to 50% of the control mean. The activities of all other complexes were normal. The parents and their daughters II-1 and II-3, aged 11 and 6 years, respectively, were healthy.

The marked reduction of complex I, III, and IV activities in patient II-2 muscle and the relative preservation of activity in complex II—the only complex that is solely encoded by the nuclear genome—suggested a defect in the synthesis of the mtDNA-encoded proteins. The ratio of mtDNA to nuclear DNA in the muscle of patient II-5, determined by real-time PCR, was 340% of the control mean, ruling out mtDNA depletion. A defect in mtDNA transcription was investigated by determination of the abundance of the transcripts of the 12S and the 16S rRNA genes and the mRNA of the heavy strand–encoded *COX2* gene in cultured fibroblasts. This analysis disclosed a slight reduction of both rRNA transcripts—12S rRNA was 70% and 16S rRNA was 65% of the control—precluding a defect in one of the ~70 mitochondrial ribosomal proteins, which would lead to a severe reduction of only one of the rRNA transcripts.[4] The *COX2* mRNA was also slightly reduced (65% of the control) in fibroblasts.

To localize the mutated gene, homozygosity mapping was performed. Genomic DNA was extracted from fibroblasts of individuals II-2 and II-4, from the cord blood of individual II-5, and from the blood samples of the parents and the two healthy sisters. All experiments involving DNA from the patients, their relatives, and healthy controls and patients' cells were performed with informed consent and were approved by the Hadassah Ethical Review Committee (number 26-160905) and by the Supreme Helsinki Committee of the Israeli Ministry of Health (number 920050420).

Homozygous regions were searched in the DNA samples of patients II-2 and II-4 with use of the Affymetrix Human Mapping 50K Array Xba240. The chip allowed genotyping of SNPs with an average distance of ~50 kb between the markers. Digestion with Xba240, ligation of the adaptor, and amplification with a generic primer that recognizes the adaptor sequence were followed by fragmentation, end labeling, and hybridization to the chip in accordance with the manufacturer's instructions. Homozygous regions >5.0 cM[8] were manually detected. Only two homozygous regions were shared by patients II-2 and II-4: on chromosome 5, 155.24–172.22 Mb (bordered by markers *rs2116739* and *rs793027*) and, on chromosome 6, 82.32–111.83 Mb (bordered by markers *rs4448140* and *rs1989574*). This latter region was narrowed by individual II-1, who shared homozygosity at the polymorphic microsatellite markers *D6S1546* and *D6S268* with her affected sibs (fig. 2). Investigation of the DNA extracted from the cord blood of patient II-5 revealed that she had a haplotype similar to that of her healthy sister II-1, by use of markers spanning the genomic region on chromosome 5 (*D5S2049, D5S422, D5S2040, D5S2050, D5S671, D5S400,* and *D5S425*). However, a haplotype identical to those of patients II-2 and II-4 was found along the chromosome 6 critical region (fig. 2). Within this 22.46-Mb region on chromosome 6, there were 103 ORFs. None of these was annotated mitochondrial, but *RARSL,* the arginyl-tRNA synthetase–like gene, was predicted mitochondrial (52% probability) by PSORT II prediction software. The gene consists of 20 exons, which code for 578 aa. We determined the sequence of each exon and its flanking intronic

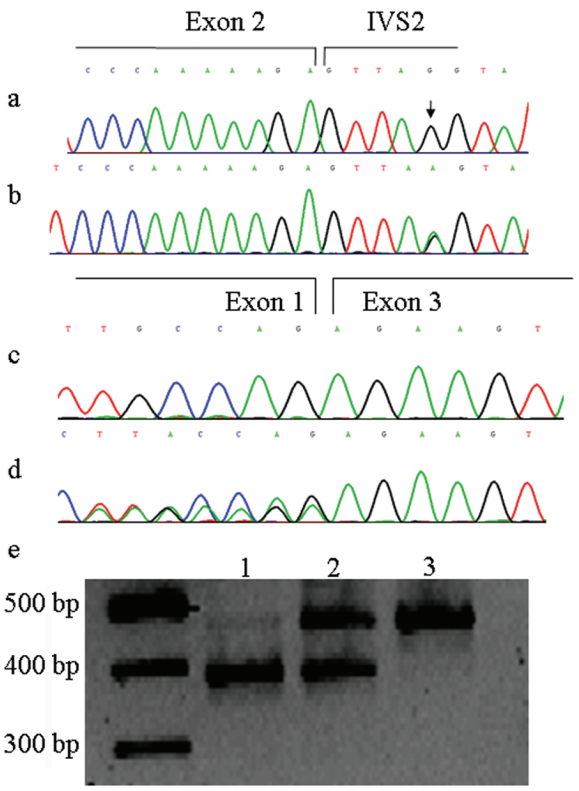

Figure 3. IVS2+5 a→g mutation, which causes exon 2 skipping. *RARS2* exon 2–IVS2 splice-junction sequence of the patient (*a*) and his mother (*b*). The mutation site is indicated by an arrow. *c, RARS2* cDNA showing exon 2 skipping at the patient sample. Exons 1 and 3 are indicated. *d, RARS2* cDNA of the mother showing heterozygosity for the wild-type and the mutant transcripts. *e,* PCR amplification products of *RARS2* cDNA encompassing the first three exons. *Lane 1,* patient; *lane 2,* mother; *lane 3,* control. A 100-bp ladder was loaded in the left lane.

sequences and identified three homozygous substitutions, K568K (aaa→aag), R291K (aga→aaa), and IVS2+5 (a→g) (fig. 3*a*). The K568K substitution did not interfere with any splicing-enhancer element, as predicted by the ESEfinder program[9]; similarly, with use of the SIFT software, the R291K substitution was not predicted to be pathogenic. The three patients were homozygous for the IVS2+5 a→g substitution, and the parents and their two healthy daughters were carriers. We sequenced the exon 2–IVS2 junction in 105 anonymous individuals of Sephardic Jewish origin and did not find any carriers for this substitution.

We next extracted total RNA from cultured skin fibroblasts of individual II-4 and from the blood of his mother, I-1, and amplified the *RARS2* cDNA (GenBank accession number NM_181406.2) in four overlapping fragments. The patient's major transcript lacked exon 2 (fig. 3*c*), but a faint, normal-sized fragment was also seen (fig. 3*e*); the mother had two transcripts of equal abundance, a normal one and a shorter one lacking exon 2. Because exon 2 consists of 74 nt, its skipping is predicted to cause a frameshift, abolishing the enzymatic activity entirely. We therefore set out to determine the extent of aminoacylation and the stability of the mitochondrial $tRNA^{Arg}$ transcript in the patient fibroblasts. Total RNA was isolated in acid conditions[10] from the patient and control fibroblasts and was separated on a 10-cm-long 10% denaturing (8 M urea and Tris/borate/EDTA) polyacrylamide gel (PAAG). Quantitative northern hybridization was performed[11] with the following oligonucleotide probes: nucleotides 10421–10441 (GAGTCGAAATCATTCGTTTTG) for the mitochondrial $tRNA^{Arg}$ and nucleotides 3275–3293 (CAGTCAGAGCTTCAATTCC) for the mitochondrial $tRNA^{Leu}$. The signals were quantified by phosphorimager (FLA 5100 [Fuji]) and were calculated by the MutiGauge Software (Fuji). The analysis of aminoacylation level in vivo was performed by hybridization with the same probes after separation of the RNA on 6.5% denaturing PAAG (20 cm long) in 0.1 M Na-acetate (pH 4.8).[10] A striking reduction in the amount of the mitochondrial $tRNA^{Arg}$ was found in the patient fibroblasts, and the $tRNA^{Arg}$:$tRNA^{Leu}$ ratio was 6% of the control. In contrast, the residual $tRNA^{Arg}$ transcript was almost fully acylated (fig. 4). These results confirm that the *RARSL* gene encodes a product with mitochondrial arginyl-tRNA synthetase activity, as predicted *in silico*.[12] The results strongly suggest that uncharged mitochondrial $tRNA^{Arg}$ transcripts become unstable. The minute amount of the wild-type *RARS2* transcript probably accounts for a residual $tRNA^{Arg}$ aminoacylation capacity in the patient fibro-

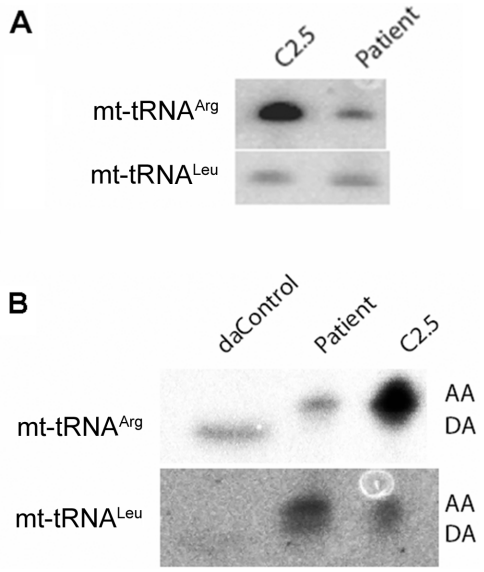

Figure 4. Quantitative and aminoacylation analysis of $tRNA^{Arg}$ in fibroblasts. *a,* Quantitative analysis of $tRNA^{Arg}$ and $tRNA^{Leu}$ in the patient and control (C2.5) by northern hybridization. Equal amounts of control and patient RNA were analyzed. *b,* Aminoacylation: results of the hybridization after separation in acid conditions. AA = aminoacylated form; DA = deacylated form. The control of fully deacylated tRNA (daControl) was obtained by incubation of the RNA at 50°C (pH 9.0) for 10 min.

blasts and for the relatively high residual respiratory-chain activity in these cells.

Until now, deficiency of the mitochondrial $tRNA^{Arg}$ synthetase had been identified only in yeast.[13] As expected, mitochondrial protein synthesis was impaired in the mutants, and the enzymatic activities of the mitochondrial respiratory-chain complexes III, IV, and V were markedly reduced.

In spite of the large number of mutations reported in mitochondrial tRNA genes, a defect in $tRNA^{Arg}$ was seldom described. An 8-year-old patient with progressive encephalopathy manifested by moderate retardation, nystagmus, delayed dysarthric speech, and clumsiness was found to carry an a→g mutation at position 10438 of the mtDNA. The mutation changed a conserved nucleotide that flanks the $tRNA^{Arg}$ anticodon sequence, and the mutant load was high in muscle (88%) and lower in blood (73%). In plasma, but not in CSF, the lactate level was slightly elevated. In muscle, complex IV activity was reduced, but complexes I, I+III, and II+III were normal.[14] Mice with a small genomic insertion in the D-loop of the mitochondrial $tRNA^{Arg}$ gene were deaf,[15] and cell lines harboring the mutation produced abnormally elevated reactive oxygen species.[16] The mechanism is unclear, since no significant difference in the relative amounts of respiratory-chain complexes estimated by western blotting was found in these cell lines. The milder phenotype in humans and mice with mitochondrial $tRNA^{Arg}$ mutations is probably the result of significant residual mitochondrial translation activity, attributed to the heteroplasmic nature of the mutation in humans and to the low pathogenicity of the mutation in mice.

A major question raised by our finding is the tissue specificity of the symptoms. Even after 16 mo of life, the symptoms of patient II-2 were still confined to the CNS, and neither the heart nor the liver, both energy-demanding tissues, were affected. The RARS2 defect is unlikely to be compensated by RARS1, because the encoded cytoplasmic arginine-tRNA synthetase lacks mitochondrial targeting sequence; furthermore, it is generally accepted that cytoplasmic tRNAs, even when properly charged, do not traverse the mitochondrial inner membrane in humans.[17,18] We therefore speculate that the leaky nature of the mutation allows for the synthesis of a small amount of wild-type *RARS2* transcript in most tissues, similar to fibroblasts. The production of the wild-type transcript in such circumstances is dependent on the splice-site score of the wild-type exon-intron junction, the presence and conservation of exonic splicing-enhancer domains, and the tissue concentration of the splicing factors. It is of note that the 3′ splice site of exon 2 of the wild-type *RARS2* gene deviates markedly from the consensus—that is, its splicing score is very low (Splice Site Score Calculation).

Very recently, mutations in the mitochondrial aspartyl-tRNA synthethase (*DARS2*) gene were reported in patients with leukodystrophy with brain stem and spinal cord involvement.[6] Similar to our patients, there was no extracerebral involvement. Nearly all the patients carried a mutation involving a stretch of T and C residues just upstream of exon 3 on one of the *DARS2* alleles. This mutation was predicted to result in skipping of exon 3, affecting, as in our case, an a priori weak splice site. We speculate that one of the splicing factors, required for the promotion of exon inclusion in both cases, is present in relatively low concentration in the brain.

Our patients presented with pontocerebellar hypoplasia (PCH) already evident at age 3 d. There are at least five types of nonsyndromic, autosomal recessive PCH. In addition, this hindbrain abnormality has been reported in association with established syndromes, congenital muscular dystrophies, lissencephaly syndromes, chromosomal abnormalities, defects in glycosylation of proteins, organic acid disorders, and mitochondrial respiratory-chain defects.[19,20] In view of the very mild mitochondrial stigmata (slight elevation of CSF lactate), it is possible that some of the autosomal recessive PCHs are in fact the result of missplicing mutations in the *RARS2* gene or in other mitochondrial aminoacyl-tRNA synthethase genes.

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Web Resources

The accession number and URLs for data presented herein are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for *RARS2* cDNA [accession number NM_181406.2])
Mitomap, http://www.mitomap.org/cgi-bin/tbl9gen.pl
PSORT II, http://psort.ims.u-tokyo.ac.jp/form2.html
SIFT, http://blocks.fhcrc.org/sift/SIFT.html
Splice Site Score Calculation at Cold Spring Harbor Laboratory, http://rulai.cshl.edu/new_alt_exon_db2/HTML/score.html

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