***RARS2* Mutations: Is Pontocerebellar Hypoplasia Type 6 a Mitochondrial Encephalopathy?**

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Abstract Mutations in the mitochondrial arginyl tRNA synthetase (*RARS2*) gene are associated with Pontocerebellar Hypoplasia type 6 (PCH6). Here we report two patients, compound heterozygous for *RARS2* mutations, presenting with early onset epileptic encephalopathy and (progressive) atrophy of both supra- and infratentorial structures. Early pontocerebellar hypoplasia was virtually absent and respiratory chain (RC) defects could not be detected in muscle biopsies. Both patients carried a novel missense mutation c.1544A>G (p.(Asp515Gly)) in combination with either a splice site (c.297+2T>G) or a frameshift (c.452_454insC) mutation. The splice site mutation induced skipping of exon 4.

These two patients expand the phenotypical spectrum associated with *RARS2* mutations beyond the first report of PCH6 by Edvardson and colleagues. We propose to classify

RARS2-associated phenotypes as an early onset mitochondrial encephalopathy, since this is more in agreement with both clinical presentation and underlying genetic cause.

Introduction

Pontocerebellar hypoplasia (PCH) was used by Barth to describe a group of prenatal onset neurodegenerative disorders with clear hypoplasia or atrophy of cerebellar and pontine structures (Barth et al. 1990; Barth 1993). These patients present with severe mental and motor retardation, progressive microcephaly, and dystonia/chorea (Namavar et al. 2011a, b). MR imaging typically shows a “dragonfly” or “butterfly” configuration of the cerebellum (Namavar et al. 2011a). New subtypes of PCH, based on differences in phenotypes and/or genotype were described over the past years, and we showed that the same genetic variant can be responsible for different forms of PCH (Namavar et al. 2011b). The current nomenclature with ten subtypes does therefore not add to the diagnostic process (Table 1).

In an effort to create more clarity in the nomenclature, we focus here on PCH6 (OMIM 611523), a genetically and biochemically distinct form of PCH. Mutations in the mitochondrial Arginine tRNA-synthetase (*RARS2*) gene underlie PCH6, and the phenotype is similar to that of patients with mitochondrial respiratory chain (RC) defects (Edvardson et al. 2007). The canonical PCH6 phenotype consists of severe early onset epilepsy, progressive global atrophy including pons and cerebellum, lactic acidosis, and/or RC defects (Cassandrini et al. 2013).

In this study, we report two patients with previously unreported mutations in *RARS2*, in whom early pontocerebellar hypoplasia was virtually absent. RC defects could

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Table 1 Overview of clinical features in patients with *RARS2*-gene mutations

Clinical feature	Number of patients affected ^a (%)
Developmental delay	29/29 (100%)
Seizures	25/29 (86%) ^b
Lactate elevated	15/22 (68%)
RC enzyme deficiency	8/17 (47%)
Early vermis hypoplasia	17/28 (61%)
Atrophy/hypoplasia of cerebellar hemispheres	24/28 (86%)
Pontine atrophy	16/28 (57%)
Cerebral atrophy	21/26 (81%)

^a Not all data are available of all patients

^b Three out of four patients without epilepsy died before the age of 2 months

not be detected in muscle biopsies taken from both patients. Based upon these two patients and a literature study, we conclude that *RARS2* mutations do not lead to a typical PCH phenotype. We therefore propose to categorize the *RARS2*-associated phenotypes as an early onset mitochondrial epileptic encephalopathy, since this is more in agreement with clinical presentation and underlying genetic cause.

Materials and Methods

Whole-Exome and Sanger Sequencing

DNA was extracted from peripheral blood samples using standard methods. In patient A *RARS2* was directly sequenced upon clinical suspicion using standard Sanger sequencing methods. In patient B, the *RARS2* mutations were identified by exome sequencing, which was performed as described before, and validated by standard Sanger sequencing methods (Neveling et al. 2013; Wortmann et al. 2015).

RT-PCR

Fibroblasts of patient A and parents were cultured on standard growth medium (DMEM (Lonza), 10% fetal calf serum (Bodinco B.V.), Penstrep (100 Units/mL Penicillin, 100 µg/mL Streptomycin, Gibco) and 10 µg/mL ascorbic acid). RNA was isolated with the RNeasy mini kit (Qiagen, Cat. No. 74106) from fibroblasts at 75% confluence or from whole blood using the PAXgene Blood RNA system (Qiagen, REF 762174, HB1051083). cDNA was synthesized with SuperScript III Reverse Transcriptase, according to the manufacturers protocol (Thermofisher). cDNA was ampli-

fied by PCR using the following M13 tailed *RARS2* primers: Exon 2–3 forward: 5'-AGCAGCTGGGATTGTAGAGA, Exon 5 reverse: 5'-CCACAATCTTCTTCTGGGGAA; exon 17 forward: 5'-TGTTTACAAGAGCCACAGTCTG; exon 19 reverse: 5'-CAGCCACTTCAGGAGGACTA. Fragment size was analyzed with agarose gel electrophoresis. DNA was extracted from gel using the silica beads method (Boom et al. 1990).

Results

Patient A

Clinical Description

Patient A is the second child of non-consanguineous parents, born at 39 weeks gestation after an uncomplicated pregnancy. Birth weight was 2,795 g (14th percentile), head circumference was 32 cm (9th percentile). Parents noted poor eye contact from birth. At 2 months of age, she was evaluated for feeding problems and jerking movements of the limbs. EEG showed an excess of slow activity with multifocal epileptiform discharges. Brain MRI showed bilateral symmetric white matter signal changes in the subcortical white matter, supratentorial atrophy, and mild vermal atrophy (Fig. 1a–d). Biochemical and metabolic investigations showed increased concentration of lactate in blood (3.2 mmol/L; normal <2.0 mmol/L), CSF (3.3 mmol/L; normal <2.3 mmol/L), and urine (lactic acid 742 µmol/L; normal <55 µmol/L). Enzyme activities of mitochondrial respiratory chain were normal in muscle, although a reduction in ATP production capacity was found (10.9 nmol/h.mUCS; reference range 15.4–30.2 nmol/h.mUCS).

At age 6 months, she continued to have epileptic seizures despite multidrug therapy, required tube feeding and had profound developmental delay. Examination showed progressive microcephaly, generalized hypertonia, and brisk reflexes. Brain MRI at age 6 months showed striking progression of infra- and supratentorial grey and white matter atrophy (Fig. 1e–g) as well as a bilateral frontoparietal and temporal subdural hygroma. Proton MR spectroscopy showed a lactate peak.

Genetic Analysis

Sanger sequencing of *RARS2* showed compound heterozygous mutations: c. 297+2T>G in intron 4 and c.1544A>G, p.(Asp515Gly) in exon 18 (NM_020320.3). Segregation analysis proved that the mutations were biallelic; father was heterozygous for the c.297+2T>G mutation and mother carried the c.1544A>G mutation.

Fig. 1 Brain MRI of patients A and B. Axial T2-weighted (a–c, e, f, i, j), sagittal (d, h, l), and coronar (g, k) T1-weighted MR images of patient 1 aged 3 months (a–d) and 6 months (e–f) and patient 2 (aged 3 months, i–l). Note the bilateral subcortical T2 signal elevation of the central region in patient 1 (a) and of the central and parietooccipital cortex and white matter in patient 2 (i, j). Between the first and second MRI, severe supratentorial atrophy develops in patient 1 (a, b vs e–g).

In patient 2, supratentorial structures also show global atrophy (i–k). Volume of pons and brainstem are normal in patient 1 (d), the vermis cerebelli is only slightly atrophic (c, d). At follow-up 3 months later, cerebellar volume appears slightly decreased with preserved pontine structures (g, h). In patient 2, there is no clear cerebellar, nor pontine atrophy (k, l)

The c.297+2T>G mutation was shown to perturb splicing by leading to a skip of exon 4 in the major *RARS2* transcript in fibroblasts of patient A and the father (Fig. 2).

Patient B

Patient B was the first child of non-consanguineous parents, born at 40 weeks of gestation after an uncomplicated pregnancy. Birth weight was 3,680 g (70th percentile). Head circumference was normal. At 3 months of age, she was admitted to the hospital for the evaluation of developmental delay and feeding problems. On examination she showed jerking movements, hypotonia, and nystagmus.

EEG showed multifocal epileptiform activity and frequently focal clonic seizures. Brain MRI showed multifocal abnormalities of the parietal and occipital cortex and white matter and diffuse supratentorial atrophy (Fig. 1i–l). MRS showed a lactate peak whereas normal lactate levels were measured in blood (1.3 mmol/L; reference range 0.7–2.3 mmol/L) and CSF (1.7 mmol/L; reference range 0.7–2.3 mmol/L). Our first suspicion with this clinical and radiological presentation was Alpers disease due to *POLG* mutations, but genetic analysis of *POLG* was negative. Enzyme activities of mitochondrial respiratory chain were normal in muscle, although a reduction in ATP production capacity was found (5.8 nmol/h.mUCS; reference range

Fig. 2 The c.297+2T>G mutation causes skipping of exon 4. **(a)** PCR amplification product of *RARS2* cDNA, exon3–5. Lane 1, patient; lane 2, father; lane 3, mother. Aberrant splicing shown in the

patient and father, suggesting exon 4 skipping. **(b)** Sanger sequencing of *RARS2* cDNA showing skipping of exon 4 in patient A

15.4–30.2 nmol/h.mUCS). She went on developing refractory status epilepticus and died 2 weeks after presentation.

Genetic Analysis

Two heterozygous mutations were identified in *RARS2* by whole-exome sequencing: c.1544A>G, p.(Asp515Gly), maternal, and c.453_454insC (p.(Asn152Lysfs*40), paternal; NM_020320.3).

In Silico Prediction of the c.1544A>G, p.(Asp515Gly) Mutation

Patient A and B both carried the c.1544A>G variant on one allele. This variant is located in exon 18 in the anticodon-binding domain, resulting in the amino acid substitution of a moderately conserved aspartic acid by glycine (p.(Asp515Gly)). In silico predictions by various programs were not uniform (SIFT: tolerated, PolyPhen HumDiv: probably damaging, with a score of 0.976, HumVar: possibly damaging with a score of 0.901). The mutation is not present in dbSNP (<http://www.ncbi.nlm.nih.gov/SNP/>), and with very low frequency (<1:10,000) in Exac (<http://exac.broadinstitute.org/>). Because the mutation is located in the Exonic Splice Enhancer domain of exon 18, an effect

on splicing was considered. However, this could not be confirmed with RT-PCR (data not shown).

Discussion

Mutations in mitochondrial aminoacyl-tRNA synthetases (mt-aaRs) have emerged as an important cause of severe early onset neurologic disorders. Aminoacyl-tRNA synthetases are essential for protein translation as they catalyze the specific attachment of amino acids to their cognate tRNA. In eukaryotic cells, protein translation takes place both in the cytoplasm and mitochondria, and aaRs are therefore needed at both locations. The nuclear genome encodes 37 aaRs genes: 17 enzymes are exclusively active in the cytoplasm, 17 are mitochondrial enzymes, and 3 aaRs are bifunctional (GlyRS, LysRS, and GlnRS). Mutations in 15 out of 17 mt-aaRs are associated with human disease. Despite the ubiquitous expression and similar function mutations in mt-aaRs genes are each associated with tissue specific but overlapping phenotypes. Mutations in *FARS2*, *VARS2*, *TARS2*, and *CARS2* are associated with early onset mitochondrial encephalopathies, whereas *RARS2* mutations were first described in a sibship with PCH and RC defects and were designated as PCH6 (Edvardson et al. 2007).

In those patients pons and cerebellum were affected first, followed by general brain atrophy. The patients were homozygous for a splice site mutation causing a skip of exon 2 and resulting in a frameshift. Only a minimal amount of normal sized fragment was seen on RT-PCR. Correspondingly, the amount of charged mitochondrial tRNA-arg was severely reduced in patient fibroblasts (Edvardson et al. 2007).

We described two patients with novel *RARS2* mutations lacking the characteristic features of PCH on MRI. At an early stage, only mild cerebellar vermal atrophy with global supratentorial atrophy was present. Follow-up in patient A showed devastating progression of neurodegeneration affecting both supra- and infratentorial structures. Both patients were compound heterozygotes and carried the same c.1544A>G, p.(Asp515Gly) mutation. The other allele in both cases was predicted to be detrimental for protein function. The p.Asp515Gly mutation is located in the anticodon-binding domain and a possible pathogenic mechanism would be diminished recognition of arginine tRNA anticodons, leading to diminished or mis-acylation of arginine to noncognate tRNAs. Although we did not investigate the pathogenicity of this mutation with functional studies, the fact that both unrelated patients with a similar phenotype share this very rare variant in combination with a deleterious mutation on the other allele is strongly indicating a pathogenic effect. Patient A carried a splice site mutation on the other allele, resulting in skipping of exon 4, which was confirmed by RT-PCR. This exon contains 84 nucleotides and a skip is therefore not disturbing the reading frame. Some residual aminoacylation activity might be conserved in this shortened protein, as is the case in many of the other mutations identified in *RARS2*. Patient B had a frameshift mutation on the other allele, leading to a premature stop codon and a truncated protein with presumably little or no residual function.

Including the two patients we report here, 29 patients with *RARS2* mutations are described in literature so far (Namavar et al. 2011a; Cassandrini et al. 2013; Rankin et al. 2010; Glamuzina et al. 2012; Kastrissianakis et al. 2013; Joseph et al. 2014; Li et al. 2015; Lax et al. 2015; Nishri et al. 2016; Ngoh et al. 2016; Alkhateeb et al. 2016). Splice site, nonsense, or missense mutations are identified throughout the *RARS2* gene, but no patients with biallelic null mutations were identified, supporting the assumption that complete abolishment of tRNA-arg would be lethal. All patients who survived the neonatal period (24/29 patients, 83%) had severe developmental delay, and all but one patient older than the age of 2 months suffered from refractory epilepsy (25/26 patients, 96%). 15 out of 22 (68%) patients (lactate levels were unknown in seven

patients) had elevated concentrations of lactate in either blood, urine, or CSF. Surprisingly, no RC defects could be detected in muscle or fibroblasts in 6 of those 15 patients (40%). Severity of cerebellar hypoplasia/atrophy was variable. MRIs made in a very early stage were either normal or showed relatively mild vermal hypoplasia, while follow-up MRIs often displayed rapidly progressive atrophy of both supra- and infratentorial structures (Cassandrini et al. 2013; Rankin et al. 2010; Kastrissianakis et al. 2013; Nishri et al. 2016). Remarkably, the basal ganglia often remained spared, a finding also endorsed by the patients we describe (Edvardson et al. 2007; Cassandrini et al. 2013; Glamuzina et al. 2012).

Recently three siblings with homozygous missense mutations in *RARS2* were reported with generalized spasticity and epilepsy, and a normal brain MRI. Unfortunately, little additional information regarding the clinical follow-up or age of brain MRI was provided (Alkhateeb et al. 2016).

Kastrissianakis and colleagues described two siblings with *RARS2* mutations with early MRIs showing marked supratentorial, instead of infratentorial, atrophy. Marked cerebellar atrophy developed later, but the pons remained relatively preserved throughout the disease course (Kastrissianakis et al. 2013). This finding is consistent with the imaging of the patients we report here, where atrophy of supratentorial structures is more striking than the pontocerebellar atrophy.

We conclude that the phenotypic spectrum associated with *RARS2* mutations has been significantly expanded beyond the initial report (Edvardson et al. 2007). As stated previously, mutations in other mt-aaRs genes have since then emerged as a cause of early onset mitochondrial encephalopathies. We therefore suggest that the name “PCH6” is no longer accurate to cover the phenotypes associated with *RARS2* mutations. A recent paper, describing two siblings with *RARS2* mutations and early onset epileptic encephalopathy without pontocerebellar hypoplasia, supports this conclusion (Nishri et al. 2016). Our proposal is to classify *RARS2*-associated phenotypes as an early onset mitochondrial encephalopathy, which is more in agreement with both clinical presentation and underlying genetic cause.

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Synopsis

PCH6 is a mitochondrial encephalopathy.

Compliance with Ethics Guidelines

Conflict of Interest

Tessa van Dijk, Fred van Ruissen, Bregje Jaeger, Richard J. Rodenburg, Saskia Tamminga, Merel van Maarle, Frank Baas, Nicole I. Wolf, and Bwee Tien Poll-The declare that they have no conflict of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki declaration of 1975, as revised in 2000. Informed consent was obtained from patients/patients' parents for being included in the study.

Author Contributions

TvD collected data, performed the RT-PCR, and wrote the first draft of the manuscript. BTPT and NIW collected patient information and critically reviewed the manuscript. FvR and RjR performed diagnostic analysis and interpretation of results. BJ, ST, and MvM collected patient information. FB supervised the laboratory procedures and results, and critically reviewed the manuscript.

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