doi:10.1093/brain/awq287 Brain 2011: 134; 143–156 | 143

BRAIN
A JOURNAL OF NEUROLOGY

Clinical, neuroradiological and genetic findings in pontocerebellar hypoplasia

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Pontocerebellar hypoplasia is a group of autosomal recessive neurodegenerative disorders with prenatal onset. The common characteristics are cerebellar hypoplasia with variable atrophy of the cerebellum and the ventral pons. Supratentorial involvement is reflected by variable neocortical atrophy, ventriculomegaly and microcephaly. Mutations in the transfer RNA splicing

Received May 28, 2010. Revised August 3, 2010. Accepted August 14, 2010. Advance Access publication October 15, 2010

endonuclease subunit genes (*TSEN54, TSEN2, TSEN34*) were found to be associated with pontocerebellar hypoplasia types 2 and 4. Mutations in the mitochondrial transfer RNA arginyl synthetase gene (*RARS2*) were associated with pontocerebellar hypoplasia type 6. We studied a cohort of 169 patients from 141 families for mutations in these genes, of whom 106 patients tested positive for mutations in one of the *TSEN* genes or the *RARS2* gene. In order to delineate the neuroradiological and clinical phenotype of patients with mutations in these genes, we compared this group with 63 patients suspected of pontocerebellar hypoplasia who were negative on mutation analysis. We found a strong correlation ($P < 0.0005$) between *TSEN54* mutations and a dragonfly-like cerebellar pattern on magnetic resonance imaging, in which the cerebellar hemispheres are flat and severely reduced in size and the vermis is relatively spared. Mutations in *TSEN54* are clinically associated with dyskinesia and/or dystonia and variable degrees of spasticity, in some cases with pure generalized spasticity. Nonsense or splice site mutations in *TSEN54* are associated with a more severe phenotype of more perinatal symptoms, ventilator dependency and early death. In addition, we present ten new mutations in *TSEN54, TSEN2* and *RARS2*. Furthermore, we show that pontocerebellar hypoplasia type 1 together with elevated cerebrospinal fluid lactate may be caused by *RARS2* mutations.

Keywords: pontocerebellar hypoplasia; TSEN; RARS2; neuroimaging; neurogenetics

Abbreviations: RARS2 = mitochondrial transfer RNA arginyl synthethase; TSEN = transfer RNA splicing endonuclease; VRK1 = vaccinia-related kinase 1

Introduction

Pontocerebellar hypoplasia represents a group of autosomal recessive neurodegenerative disorders with prenatal onset, predominantly affecting growth and survival of neurons in the cerebellar cortex, the dentate, inferior olivary and ventral pontine nuclei. The variable involvement of supratentorial structures includes ventriculomegaly, neocortical atrophy and microcephaly. Radiologically and pathologically, all subtypes are characterized by hypoplasia and variable atrophy of the cerebellum and pons.

Six subtypes of pontocerebellar hypoplasia have so far been identified. Type 1 (MIM 607596) is characterized by additional loss of motor neurons in the spinal cord, morphologically similar to the hereditary spinal muscular atrophies (Norman, 1961; Goutieres *et al.*, 1977; Barth, 1993, 2000). Recently, an association with the vaccinia-related kinase 1 gene (*VRK1*) was reported in a single family with a mild variant of pontocerebellar hypoplasia type 1 (Renbaum *et al.*, 2009). In pontocerebellar hypoplasia type 2 (MIM 277470, 612389, 612390), the distinctive feature is dyskinesia and/or dystonia and, more rarely, pure spasticity (Barth *et al.*, 1995). Pontocerebellar hypoplasia type 4 (MIM 225753), previously known as olivopontocerebellar hypoplasia, has a more severe course with prenatal onset of clinical symptoms such as polyhydramnios and contractures. Early postnatal death is often reported, usually due to primary respiratory insufficiency. Typical for pontocerebellar hypoplasia type 4 are the C-shaped inferior olives, indicating an earlier prenatal onset than seen in type 2 (Albrecht *et al.*, 1993; Barth *et al.*, 2007). Neuropathologically, pontocerebellar hypoplasia type 4 and type 2 both display the fragmentation of the cerebellar dentate nuclei. Mutations in the transfer RNA splicing endonuclease subunit gene *TSEN54* are responsible for pontocerebellar hypoplasia type 2 and 4 in most European cases. All mutations identified in type 2 cases are missense mutations (*TSEN54, TSEN2, TSEN34*). In pontocerebellar hypoplasia type 4, nonsense and missense mutations have been identified together (*TSEN54*) (Budde *et al.*, 2008).

Pontocerebellar hypoplasia types 3, 5 and 6 (MIM 608027, 610204, 611523, respectively) are rare forms of pontocerebellar hypoplasia. Type 3, also known as cerebellar atrophy with progressive microcephaly, is associated with optic atrophy, seizures, hypotonia and short stature (Rajab *et al.*, 2003; Durmaz *et al.*, 2009). Type 3 is mapped to chromosome 7q [markers D7S802 and D7S630 define the borders of the region (Durmaz *et al.*, 2009)] but no gene has yet been identified. Type 5 is characterized by intra-uterine seizure-like activity and a predominantly affected vermis (Patel *et al.*, 2006). No associated locus has been identified. Type 6 has been reported in two families: one with associated mitochondrial respiratory chain abnormalities and the other with progressive encephalopathy, oedema, hypsarrhythmia and optic atrophy-like features. Mutations in the nuclear encoded mitochondrial arginyl transfer RNA synthetase gene (*RARS2*) have been identified (Edvardson *et al.*, 2007; Rankin *et al.*, 2010).

In this study, we investigated a cohort of 169 patients (141 families) who were referred to our laboratory for molecular genetic testing due to pontocerebellar hypoplasia. We screened the coding regions for *TSEN54, TSEN2, TSEN34, TSEN15, RARS2* and *VRK1* mutations. In order to define the phenotypical spectrum in patients with the common *TSEN54* mutation, brain MRI and the clinical phenotype of patients with the common *TSEN54* mutation were compared with patients where we did not find a mutation. Here we show that on MRI, a dragonfly-like pattern of the cerebellum is significantly associated with the common homozygous p.A307S mutation in *TSEN54* (100%). The common mutation in *TSEN54* is associated with progressive microcephaly, severe lack of motor development, dyskinesia and/or dystonia, central visual impairment and impaired swallowing. These findings, together with a dragonfly-like cerebellum, are highly specific for pontocerebellar hypoplasia type 2 with the common mutation and can be implemented in the clinical and molecular diagnosis of type 2 patients. Compound heterozygotes with a nonsense mutation and a missense mutation in *TSEN54* are associated with

a more severe phenotype with pre- and peri-natal onset of symptoms, such as polyhydramnios, contractures, dependence on mechanical ventilation (>1 day after birth) and early death.

Patients and methods

Patient cohort

For this study, we selected a group of 169 patients (141 families) referred for molecular genetic testing of pontocerebellar hypoplasia-associated genes. All patients who took part in our study were diagnosed with pontocerebellar hypoplasia by referring clinicians. Using these non-strict selection criteria, we aimed to include typical and atypical patients that represent the spectrum of cases submitted to laboratories performing genetic testing. Blood or genomic DNA samples were provided through neurology, paediatric and clinical genetic departments worldwide. Samples were submitted to our department for diagnostics and informed consent was obtained by referring physicians.

Genetic analysis

The coding regions and exon-intron boundaries of *TSEN54*, *TSEN34*, *TSEN2*, *TSEN15, RARS2* and *VRK1* were sequenced on both strands. If DNA levels were not sufficient, DNA was amplified with the GenomiPhi V2 DNA Amplification kit (GE Healthcare, Waukesha, USA) according to the manufacturer's protocol. For 28 cases, DNA from affected siblings was available. All coding regions and exon–intron boundaries for ≥1 case per family were sequenced. Once a mutation was found, it was verified in the affected sibling. Primer pair sequences, polymerase chain reaction and sequence conditions are available upon request. Polymerase chain reaction products were directly sequenced using BigDye Terminator sequencing kit and ABI PRISM 3730 DNA analyser (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Sequences were analysed using the CodonCode Software version 3.0.1 (Dedham, MA, USA). Possible variants were confirmed by re-sequencing a new polymerase chain reaction product. When available, DNA from parents was analysed to confirm segregation. One hundred and sixty-seven control chromosomes (88 individuals) were screened to exclude polymorphisms. The study design is shown in Fig. 1.

Clinical analysis

Detailed clinical information was available for 85 patients. This included pre- and peri-natal morbidity and disease course (Table 1). Additional information requested by questionnaire included specific questions on (progressive) microcephaly, motor achievements, visual and feeding behaviour, speech and contractures.

Magnetic resonance imaging analysis

Complete magnetic resonance neuroimages (coronal, sagittal and axial sections) were available for 50 patients. Prior to DNA analysis, coronal, sagittal and axial MRIs were qualitatively analysed (by P.G.B. and B.T.P.T.). Cerebellum, pons, cerebral cortex, ventricles and myelination were analysed and divided into different categories (for further explanation see Tables 2 and 3 and Fig. 2A–D).

Statistical analysis

Fisher's exact test and Chi-square test for trend were used to test whether there were significant phenotypical differences between the different mutation groups and the group in whom no mutations were identified.

Results

We identified disease-causing mutations in 106 of the 169 patients, representing a mutation frequency of 62.7%. One hundred patients (59.2%) had a disease causing mutation in *TSEN54*. Eighty-eight of these patients were homozygous for the common mutation (p.A307S) in *TSEN54* (52.1%). MRI scans were available for evaluation from 50 individuals. Twenty of these 50 patients had the common mutation, in 13 cases we identified a rare mutation and in the remaining 17 of the 50 imaged cases we did not identify a mutation. All mutations that we identified were verified in 176 control chromosomes and none of them were found homozygous in healthy individuals. We also analysed, but did not identify, mutations in the candidate gene *TSEN15* and the *VRK1* gene.

TSEN54 common mutation

Neurological phenotype

Eighty-eight of 169 patients were homozygous for the common mutation (p.A307S) in *TSEN54* (52.1%). Due to the size of this group, we were able to redefine the phenotype in detail and compare with patients without mutations (Table 1). Pre- and peri-natal complications, such as polyhydramnios and contractures, were rare in patients with the common mutation. Neonatal irritability (jitteriness and/or clonus) and dyskinesia and/or dystonia were

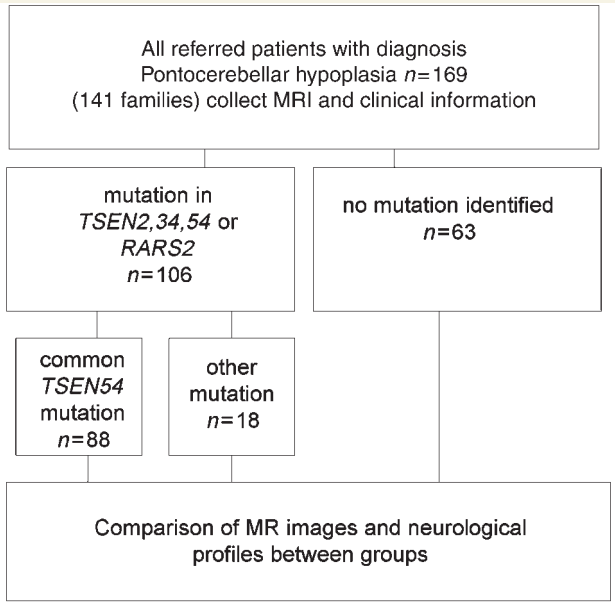

Figure 1 Study design.

Table 1 Clinical symptoms in patients with the common mutation and in patients in whom no mutation was identified

	Homozygous for *TSEN54* p.A307S		No mutation identified	
	(*n* = 88)	%	(*n* = 63)	%
Prematurity (<38 weeks gestation)	11/63	17.5	10/29	34.5
Polyhydramnios	3/67	4.5	5/29	17.2
Jitteriness, clonus	45/50[a]	90	15/25	60
Congenital contractures	1/62	1.6	3/27	11.1
Microcephaly <−2 SD	73/73	100	40/42	95.2
Progressive microcephaly	67/67[a] 1 ed	100	21/26 2 ed	80.8
Dyskinesia/dystonia	69/72[a,b]	95.8	20/34 3 ed	58.8
Pure spasticity	2/72[b]	2.8	3/32 3 ed	9.4
Impaired swallowing	68/69[a]	98.6	18/30	60.0
Central visual impairment	52/60[a] 2 ed	86.7	9/20 2 ed	45.0
Primary optic atrophy	0/59[a,c]	0	5/18	27.7
Epileptic seizures, all types	44/54	81.5	17/23 1 ed	73.9
Mechanical ventilation >1 day after birth	4/64	6.3	8/27 1 ed	29.6
Median age at last examination	34.5 months (58 patients)		28 months (34 patients)	
Survival range (median age of death)	2.5 weeks to 31 years[d] (50 months, 18 patients)		1 day to 22 years (6 months, 13 patients)	
Hand control[e]	43 Nn[a] 10 I 2 ed		6 Nn 5 I 7 G 3 ed	
Postural antigravity control[f]	28 Nn[a] 17 S 1 T 1 Us 2 ed		9 Nn 5 S 1 T 1 H 7 Us 3 ed	

The number of patients with a symptom is given relative to all patients with information on this symptom. Percentages indicate the proportion of the patients with a symptom relative to all patients with symptom information. ed = early death.
a Significantly associated with the common mutation group, compared to cases where no mutation was identified. The absence of primary optic atrophy was also significantly associated with the mutation.
b One case did not exhibit dyskinesia, dystonia and spasticity, however, this case was <3 months at the time of the examination.
c One case had optic atrophy secondary to glaucoma. Primary optic atrophy was not reported in any of these cases.
d Patient is alive.
e Hand control: G = grasping; I = intentional; Nn = none.
f Postural antigravity control: H = with hip support; Nn = none; S = with shoulder support; T = with high thoracic support; Us = unsupported sitting.

Table 2 MRI findings in patients with the common mutation and in patients in whom no mutations were identified

	TSEN54 p.A307S p.A307S (*n* = 20)					No mutation identified (*n* = 17)				
Morphological stage	**0**	**1**	**2**	**3**	**4**	**0**	**1**	**2**	**3**	**4**
Cerebellar hemispheres[a]	0	20	0	0	0	0	6	5	0	6
Pons[b]	0	4	16	–	–	2	12	3	–	–
Vermis folial atrophy[c,d]	9	10	0	–	–	6	6	3	2 afolia	–
Cerebral cortex[e]	12	4	4	0	–	11	2	3	1	–
Ventricles[f]	6	6	8	–	–	7	4	6	–	–

Reference sequences are NM_207346.2, NM_025265. 2, NM_024075.2 and NM_020320.3 (for *TSEN54, TSEN2, TSEN34* and *RARS2,* respectively).
a Based on coronal images, the cerebellar hemispheres were distinguished into different groups: (1) a dragonfly type, with flattened cerebellar hemispheres ('the wings') and a relatively preserved vermis ('the body'); (2) a butterfly type, with a small cerebellum where the proportional size of hemispheres and vermis is preserved; (3) a postnatal atrophy type; and (4) all cases that cannot be categorized under (1)–(3).
b Pons was scored as normal (0), attenuated (1) or flat (2).
c Vermal folia were scored as normal (0), atrophy (1) or severe atrophy (2).
d In one case it was not possible to determine the degree of folial atrophy in the vermis.
e Cerebral cortex was scored as normal (0), mild atrophy with visible sulci (1), moderate or severe atrophy (2) or delayed maturation with immature aspect (3).
f Ventricles were scored as normal (0), anterior > posterior (1) or general dilatation (2).

associated with the presence of the common mutation ($P < 0.005$ and < 0.0001, respectively). Impaired swallowing contributing to failure to thrive and requiring nasogastric tube feeding or percutaneous endoscopic gastrostomy is frequently seen in patients with the common mutation ($P < 0.0001$). Progressive microcephaly becomes more evident with increasing age and was associated with the common mutation ($P < 0.005$) (Fig. 3, Roche *et al.*, 1987).

Impaired hand and head control and central visual impairment in the absence of primary optic atrophy were also strongly associated with the presence of the common mutation ($P < 0.0005$). Primary optic atrophy was ultimately used as an exclusion criterion for the common mutation, as none of the patients with this mutation displayed such a phenotype.

Taking all aforementioned criteria together, these characteristics fit with a pontocerebellar hypoplasia type 2 phenotype.

Seizures were often reported, but were also present in the majority of patients in whom we did not identify a mutation ($P = 0.542$).

Table 3 MRI findings in patients with a rare mutation

Family code	Gene	Mutation	Age (mean 12 weeks)	Cerebellar hemispheres[a]	Pons[b]	Vermis folial atrophy[c]	Cerebral cortex[d]	Ventricles[e]	Unusual findings
Ch1 II.1	*TSEN54*	p.E60AfsX109 p.A307S	8 days	4	1	1	3	0	Generally small cerebellum
Br1 II.2	*TSEN54*	p.S93P p.A307S p.A307S	6 days	1	1	1	3	2	
Se2 II.2	*TSEN54*	Splice site mutation p.A307S	2 days	1	1	1	3	0	
Dh1 II.1	*TSEN54*	p.G124V p.A307S	7 months	1	2	0	0	1	
Vi5 II.1	*TSEN54*	p.G124V p.A307S	10 months	3	2	0	1	1	
Ut4 II.1	*TSEN54*	p.Q246X p.A307S	3 days	1	2	0	3	2	Abnormal white matter signal cerebellar hemispheres
Nu1 II.1	*TSEN54*	p.A307S p.Q343X	1–2 weeks	1	2	2	3	2	
Lj1 II.1	*TSEN54*	p.A307S p.Y513D	2 days	1	2	2	3	0	
Bd1 II.1	*TSEN54*	p.A307S p.P318QfsX23	10 months	1	2	0	0	1	
Gn6 II.1	*TSEN54*	p.A307S p.R353GfsX81	6 weeks	4	2	2	3	2	Generally small cerebellum
Pa1 II.3	*TSEN2*	p.Y309C splice site mutation	10 days	2	2	0	1	2	
Hg1 II.1	*TSEN34*	p.R58W p.R58W	1 year	2–3	1	1	0	2	
Ex1 II.1	*RARS2*	p.Q12R p.M342V	13 months	2	1	0	1	2	

Reference sequences are NM_207346.2, NM_025265. 2, NM_024075.2 and NM_020320.3 (for *TSEN54*, *TSEN2*, *TSEN34* and *RARS2*, respectively).

a Based on coronal images, the cerebellar hemispheres were distinguished into different groups: (1) a dragonfly type, with flattened cerebellar hemispheres ('the wings') and a relatively preserved vermis ('the body'); (2) a butterfly type, with a small cerebellum where the proportional size of hemispheres and vermis is preserved; (3) a postnatal atrophy type; and (4) all cases that cannot be categorized under (1)–(3).

b Pons was scored as normal (0), attenuated (1) or flat (2).

c Vermal folia were scored as normal (0), atrophy (1) or severe atrophy (2).

d Cerebral cortex was scored as normal (0), mild atrophy with visible sulci (1), moderate or severe atrophy (2) or delayed maturation with immature aspect (3).

e Ventricles were scored as normal (0), anterior > posterior (1) or general dilatation (2).

A wide range of life expectancy was reported. While one child died at the age of 2.5 weeks, one patient is now alive at 31 years of age.

We previously established the allele frequency of the common p.A307S mutation in German and Dutch individuals ($n = 730$) and identified 6 heterozygote genotypes (Budde *et al.*, 2008). Effects of the common mutation on protein function was predicted with Alamut software 1.5 (Interactive Biosoftware, Rouen France) (Tables 5 and 6) (Budde *et al.*, 2008).

Magnetic resonance imaging analysis

Based on coronal images, we divided the cerebellar hemisphere pathology into four different categories (Table 2, Fig. 2A–D): (1) a dragonfly type, with flattened cerebellar hemispheres ('the wings') and a relatively preserved vermis ('the body'); (2) a butterfly type, with a small, normally proportioned cerebellum; (3) a postnatal atrophy type; and (4) the remainder of cases that cannot be categorized in (1–3) above. None of the images showed normal hemispheres. The dragonfly-like cerebellar hemispheres were significantly associated with the presence of the common mutation compared to cases where no mutation was identified ($P < 0.0005$; Fig. 2A and B). All 20 cases with the common mutation had dragonfly-like hemispheres. Six of the 17 cases of the group without a mutation also showed this MRI phenotype.

The shape of the pons was divided into three different categories: (1) normal; (2) attenuated; or (3) flat. The degree of attenuation of the pons was significantly associated with the presence of the common mutation ($P < 0.0005$; Fig. 4A).

The shape of the cerebellar folia in the vermis, the atrophy and/or maturity of the cerebral cortex and the size of the ventricles were not significantly associated with patients with a common mutation, compared to patients in whom no mutation was identified ($P = 0.213$, 0.871 and 0.573, respectively). The vermal folia and the cerebral cortex were relatively preserved. Also, no correlation was found between ventricular size and the presence of the common mutation. Generally, myelination of the cerebral hemispheres was delayed. No signs of demyelination were found.

Additional findings in the common mutation group

In eight patients (40%), mild to severe cerebral cortical atrophy was seen on MRI (Table 2, Fig. 5A). This phenomenon correlates with increasing age, suggesting that cortical atrophy might develop in all cases with pontocerebellar hypoplasia type 2.

One patient (Am1b II.1) had vermal and cerebellar hemispheric cysts (Fig. 5B and C). Autopsy of this patient revealed that the cysts were destructive (Barth *et al.*, 2007). We did not observe cysts in affected family members of this patient. However, cysts were found in one unrelated case where we did not find a mutation.

Genetic, clinical and neuroradiological findings in cases with a rare mutation

In 18 patients (17 families) of our cohort of 169 patients, we identified a rare combination of mutations in *TSEN54*, *TSEN34*, *TSEN2* or *RARS2*. In 10 patients we observed a combination of mutations not previously published (Table 4). Effects of these

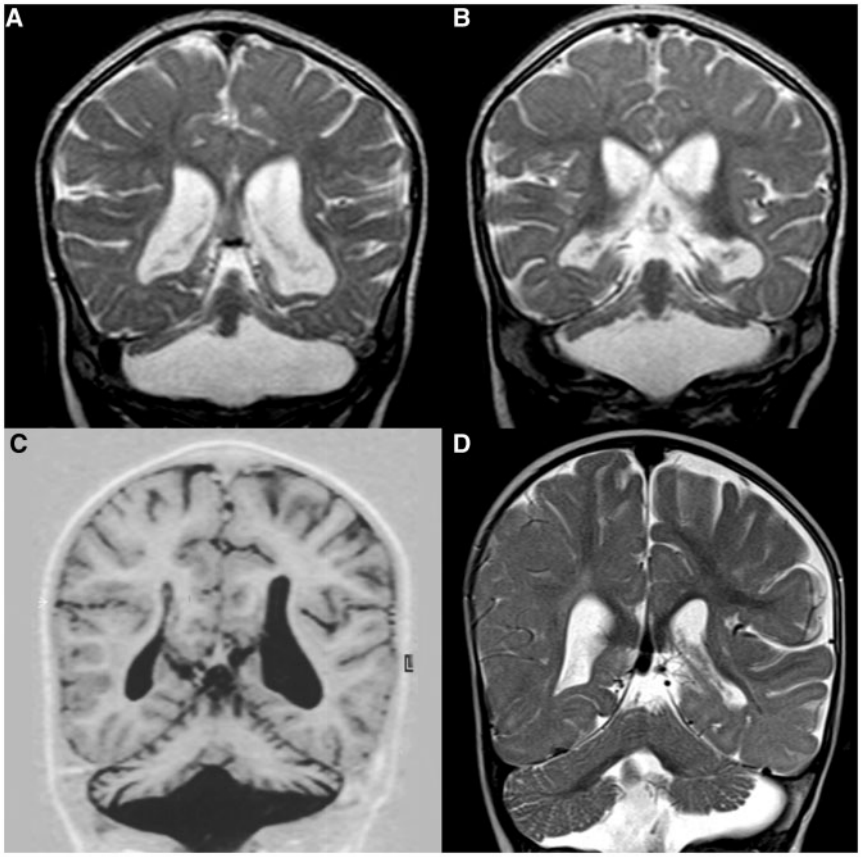

Figure 2 Examples of MRI scores of cerebellum. (**A** and **B**) Cerebellar hemisphere score 1: dragonfly type, with flat cerebellar hemispheres and a relatively spared cerebellar vermis (coronal sections, T_2-weighted, 9 months). Mild atrophy of the cerebral cortex with visible sulci. Homozygous for common *TSEN54* mutation. (**C**) Cerebellar hemisphere score 2: butterfly type, with hypoplastic cerebellar hemispheres. Decrease in size of the vermis is proportional to the diminution in size of the hemispheres (coronal section, T_1-weighted, 7 years). In addition, cerebellar cortical atrophy is seen, as well as mild atrophy of the cerebral cortex with visible sulci. No mutation identified. (**D**) Cerebellar hemisphere score 3: postnatal atrophy-like on the right side, combined with mild cerebellar hypoplasia on the left (coronal section, T_2-weighted, 10 months). In addition there is cerebral cortical atrophy with visible sulci. Heterozygote for uncommon *TSEN54* mutation (p.G124V) plus heterozygote for common *TSEN54* mutation.

mutations on protein function or transcription were predicted with Alamut software 1.5 (Interactive Biosoftware, Rouen France) (Tables 5 and 6).

Severe *TSEN54* mutations

Nine patients were compound heterozygote and we classified their combination of mutations as severe *TSEN54* mutations (Table 4). Of these, five (Ch1, Bd1, Ut4, Nu1, Gn6) carried a nonsense mutation plus a common mutation. Three individuals (Se1, Se2, Us15) carried a splice site mutation plus a common mutation. No RNA was available to test for splicing errors. *In silico* analysis suggests loss of a splice donor site in two patients (Se1 and Se2) and skipping of exon 10 in one patient (Us15). Segregation was confirmed by testing parental DNA. One patient (Br1) had three mutations in *TSEN54*; on one allele she had the common p.A307S mutation; on the second allele she also carried the p.A307S mutation plus another missense mutation, p.S93P (Budde *et al.*, 2008).

All cases had severe congenital symptoms. One patient (Se2) was antenatally diagnosed with cerebellar hypoplasia (gestational age of 19 weeks). In five patients, polyhydramnios was reported ($P < 0.05$) (Table 4). Contractures were reported in six patients ($P < 0.005$) (Table 4). All but one case (Bd1) were dependent on mechanical ventilation ($P < 0.01$). Severe myoclonus was reported in all of them and all but one died in their first year (range: 2 days–16 months, median age at death: 12 days). Additionally, one case (Gn6) is still alive (age at latest examination 6 weeks). In summary, patients who were compound heterozygote for a missense mutation plus nonsense or splice site mutations in *TSEN54* were more severely affected than patients homozygous for the common mutation. These patients fit a pontocerebellar hypoplasia type 4 phenotype, which includes early death, prolonged dependency on mechanical ventilation following birth, severe myoclonus, contractures and increased frequency of polyhydramnios. MRIs of these patients reveal pathology comparable to patients with the common mutation in *TSEN54* (Tables 2 and 3, Fig. 6A–C). The cerebellar hemispheres are similar, however the vermis is more frequently affected. The most striking difference, compared to the common mutation group and to patients without a mutation, is the immaturity of the cerebral cortex in this

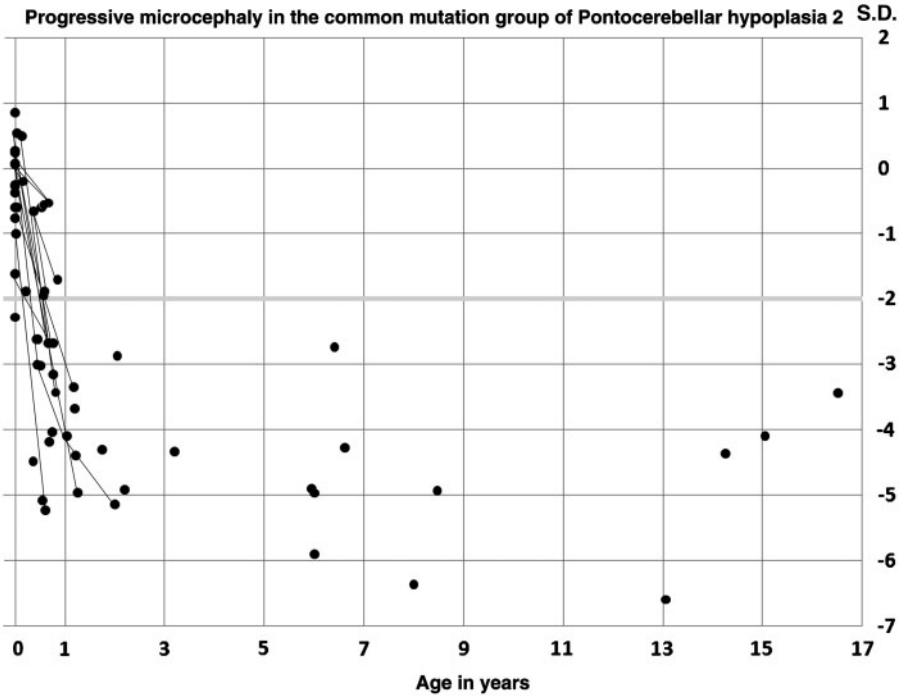

Figure 3 Progressive microcephaly in patients with the common mutation. Frontal-occipital circumference was measured in 38 cases (61 measurements). Measurements of individual patients are connected within the first 3 years of life to illustrate rates of progression. Reference measurements were used from Roche *et al.* (1987).

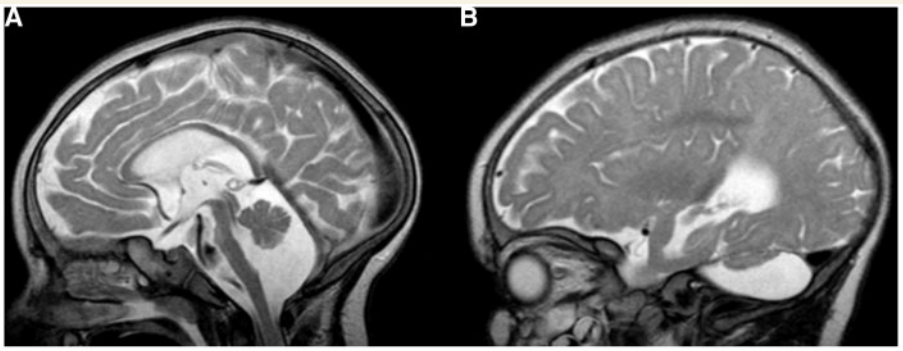

Figure 4 Typical MRI seen in the common mutation group. Flat ventral surface of the pons, cerebellar hemispheric and mild vermal hypoplasia. In this case without significant folial atrophy (sagittal sections, T_2-weighted, 9 months). The corpus callosum is too thin. Homozygous for common *TSEN54* mutation.

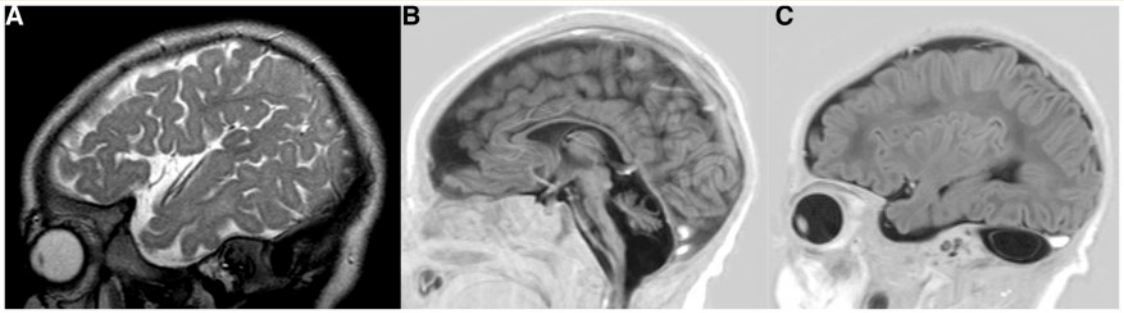

Figure 5 Additional findings in patients with the common mutation (**A**). Neocortical atrophy (sagittal view, T_2-weighted, 9 months). Homozygous for common *TSEN54* mutation. (**B** and **C**) Cerebellar vermal (**B**) and hemispheric cyst (**C**). Mid and lateral sagittal sections from Patient Am1b II.1 (T_1-weighted Inversion Recovery, 12 months). Homozygous for common *TSEN54* mutation.

Table 4 Clinical symptoms in patients with rare mutations in *TSEN54*, *TSEN34*, *TSEN2* or *RARS2*

Family code	Ch1 II.1[d]	Br1 II.2	Se2 II.2	Dh1 II,1	Vi5 II.1	Ut4 II.1	Nu1 II.1	Se1 II.1	Us15 II.1	Lj1 II.1	Bd1 II.1	Gn6 ii.1	Le1 II.1	Pa1 II.1 / II.3	Hg1 II.1	Ex1 II.1[c]	Sf1 II.1
Gene	*TSEN54*	*TSEN54*	*TSEN54*	*TSEN54*	*TSEN54*	*TSEN54*	*TSEN54*	*TSEN54*	*TSEN54*	*TSEN54*	*TSEN54*	*TSEN54*	*TSEN2*	*TSEN2*	*TSEN34*	*RARS2*	*RARS2*
Mutation (allele A)	p.E60A fsX109	p.S93P p.A307S	p.A95A[e]	p.G124V	p.G124V	p.Q246X	p.A307S	p.A307S	p.A307S	p.A307S	p.A307S	p.A307S	p.Y309C	p.Y309C	p.R58W	p.Q12R	p.Q12R
Mutation (allele B)	p.A307S	p.A307S	p.A307S	p.A307S	p.A307S	p.A307S	p.Q343X	p.P417P[e]	c.1430+ 2T>A[e]	p.Y513D	p.P318Q fsX23	p.R353G fsX81	p.Y309C	c.960+ 1delGTAAG[e]	p.R58W	p.M342V	c.110+ 5A>G[e]
Pregnancy duration (weeks)	31	At term	34 5/7	40	u	38	35	38	39	38 4/7	37 6/7	31	at term	39 4/7 / 39	u	at term	38
Polyhydramnios	+	+	+	–	–	–	+	+	–	+	–	–	–	– / –	–	–	–
Jitteriness/clonus	+	+	+	–	u	+	+	+	+	+	+	+	u	+ / +	u	+	–
Congenital contractures	+	–	+	–	–	+	–	+	+	+	–	+	–	– / –	–	u	+
Microcephaly	u	+	+	+	+	+	+	+	u	+	+	+	+	+ / +	+	+	–
Progressive microcephaly	u	+	ed	+	+	ed	+	ed	u	+	+	+	+	+ / +	+	+	–
Dyskinesia/dystonia	ed	–	ed	+	+	ed	–	ed	ed	ed	–	+	+	+ / –	+	–	ed
Pure spasticity	ed	–	ed	–	–	ed	+	ed	ed	ed	–	–	–	– / –	–	–	ed
Impaired swallowing	u	+	+	–	+	+	+	u	+	+	+	+	–	+ / +	–	+	u
Central visual impairment	u	+	ed	–	+	ed	+	ed	ed	ed	+	u	+	+ / u	+	+	+
Primary optic atrophy	u	+	u	–	u	u	–	u	u	u	u	–	–	u / u	–	u	–
Epileptic seizures, all types	u	u	–	+	–	u	+	+	u	–	+	+	+	+ / –	+	+	u
Mechanical ventilation >1 day after birth	+	+	+	–	–	+	+	ed	+	–	–	+	–	– / –	–	–	+
Age at latest examination	9 d	u	6 d	4 yr	11 mo	2 d	8 mo	1 d	2 wk	3 wk	11 mo	6 wk	3 yr	4 yr / 1 mo	4 yr	2 yr	5 d
Age at death	9 d	3 mo	6 d	Alive	Alive	2 d	16 mo	1 d	2 wk	6 wk	12 mo	Alive	Alive	Both alive	alive	alive	6 d
Hand control[a]	ed	Nn	ed	G	I	ed	Nn	ed	ed	Nn	Nn	u	G	Nn/Nn	Nn	u	ed
Postural antigravity control[b]	ed	Nn	ed	Us	Nn	ed	Nn	ed	ed	Nn	Nn	u	Us	Nn/Nn	Nn	S	ed

Reference sequences are NM_207346.2, NM_025265. 2, NM_024075.2 and NM_020320.3 (for *TSEN54*, *TSEN2*, *TSEN34* and *RARS2*, respectively).
Plus = Yes; Minus = No; u = unknown; ed = early death; wk = weeks; mo = months; yr = years.
a Hand control: G = grasping; I = intentional; Nn = none.
b Postural antigravity control: H = with hip support; Nn = none; S = with shoulder support; T = with high thoracic support; Us = unsupported sitting.
c Patient from Rankin *et al.* (2010).
d DNA of patient was no longer available, therefore the genotype of the patient was predicted from sequencing parental DNA.
e Splice site mutation.

Table 5 Missense and nonsense mutations in pontocerebellar hypoplasia

Gene	Nucleotide position	Protein position	Grantham score	Polyphen	Conservation amino acid	Other[a]
TSEN54	c.919G>T	p.A307S	99	Benign	Moderate conserved	–
TSEN54	c.371G>T	p.G124V	109	Probably damaging	Highly conserved	–
TSEN54	c.953delC	p.P318QfsX23	–	–	–	Truncated protein
TSEN54	c.736C>T	p.Q246X	–	–	–	Truncated protein
TSEN54	c.1537T>G	p.Y513D	160	Probably damaging	Highly conserved	–
TSEN54	c.1027C>T	p.Q343X	–	–	–	Truncated protein
TSEN54	c.178_215del	p.E60AfsX109	–	–	–	Truncated protein
TSEN54	c.277T>C	p.S93P	74	Possibly damaging	Highly conserved	–
TSEN54	c.1056_1057del	p.R353GfsX81	–	–	–	Truncated protein
TSEN34	c.172C>T	p.R58W	101	Probably damaging	Moderate conserved	–
TSEN2	c.926A>G	p.Y309C	194	Probably damaging	Moderate conserved	–
RARS2	c.35A>G	p.Q12R	43	Possibly damaging	Moderate conserved	–
RARS2	c.1024A>G	p.M342V	21	Probably damaging	Highly conserved	–

a Predictions were made with Alamut software 1.5 and Grantham score (Grantham, Science 1974).

Table 6 Splice site mutations in pontocerebellar hypoplasia

Gene	Nucleotide position	Protein position	Splice site mutation[a]
TSEN54	c.1251A>G	p.P417P	Loss of a splice donor site
TSEN54	c.1430+2T>A	–	Skip of exon 10
TSEN54	c.285G>C	p.A95A	Loss of a splice donor site
TSEN2	c.960+1delGTAAG	–	Skip of exon 7
RARS2	c.110+5A>G	–	Skip of exon 2[b]

a Predictions were made with Alamut software 1.5 and Grantham score (Grantham, Science 1974).
b Edvardson *et al.* (2007).

severely affected group ($P = 0.0003$ and 0.0012, respectively, Fig. 6A–C). In seven cases with pontocerebellar hypoplasia type 4 (Ch1, Se2, Ut4, Br1, Nu1, Us15, Gn6), the maturation of the cerebral cortex was underdeveloped for postconceptional age, with increased volume of extracerebral CSF and exceptionally large midline cava, most likely due to the lack of growth of the cerebral hemispheres.

Autopsy revealed similar pathology to the MRI. Olivopontocerebellar hypoplasia was observed in Patient Ut4 (Patient 7 and Patient Ut4 in Barth *et al.*, 2007 and Budde *et al.*, 2008, respectively), which included severely reduced folial development in the cerebellar hemispheres and the horseshoe appearance of the inferior olivary nucleus. Autopsy of Se2 revealed a similar pathology with additional severe cerebral immaturity and atrophy most pronounced in the frontal lobes. Motor neurons of the spinal anterior horns were intact.

Rare *TSEN54* missense mutations

Three patients were compound heterozygotes for missense mutations in *TSEN54* (Dh1, Vi5 and Lj1; Table 4). Two of these patients (Dh1, Vi5) carry the same combination of mutations (p.G124V and p.A307S). Compared to the common mutation group, Patient Dh1 is relatively well. She is able to sit unsupported, grasp, smile and socially interact with her parents. From this perspective it will be interesting to follow up Patient Vi5, who is still young (aged 11 months at last examination). With regard to her MRI, Patient Dh1 (Table 3) is similar to the common mutation group, which suggests that the degree of infratentorial involvement may not relate to postural motor control, central visual impairment and intellectual performance. The MRI of Patient Vi5 shows postnatal atrophy of the cerebellar hemispheres (Table 3, Fig. 2D).

Patient Lj1 was compound heterozygote for the common mutation and the p.Y513D change. She had an early death pontocerebellar hypoplasia type 4 phenotype, similar to the severe *TSEN54* mutation group. This early postnatal death phenotype is associated with more severely affected cerebellar hemispheres, pons, vermis, cerebral cortex and ventricles than observed in cases with the common mutation (Table 3, Fig. 6A–C).

TSEN2 mutations

In two families (Table 4; Le1, Pa1) missense and splice site mutations in *TSEN2* were identified. In one patient (Le1, Budde *et al.*, 2008) with a homozygous mutation in *TSEN2,* motor control (recorded as unsupported sitting and the ability to grasp objects) was better in comparison to the common *TSEN54* mutation group. Two siblings (Pa1 II.1 and II.3) match the common mutation group phenotype in all regards. Pa1 II.1 and II.3 carry a splice site mutation on one allele (c.960+1delGTAAG) and a missense mutation on the other allele (p.Y309C).

TSEN34 mutations

One patient (Table 4, Hg1) with a homozygous missense mutation in *TSEN34* was similar in symptomatology to the common *TSEN54* mutation group, except for the absence of dysphagia (Budde *et al.*, 2008). MRIs of this case show mild involvement of cerebellum and pons (similar to Fig. 2D).

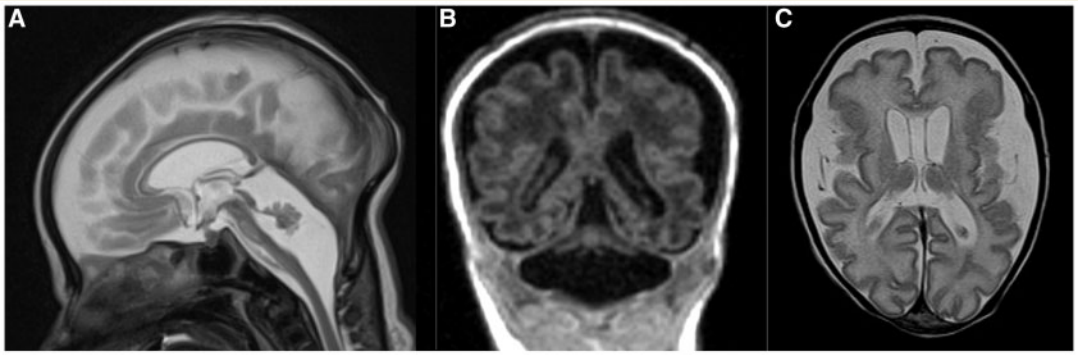

Figure 6 Typical findings in severe mutation group (pontocerebellar hypoplasia type 4 phenotype). (**A**) Showing flat lower surface of the pons and more severe involvement of vermis with extreme hypoplasia and moderate atrophy (midsagittal section, T_2-weighted, 31+5 weeks). Heterozygote for nonsense mutation in *TSEN54* (p.R353GfsX81) plus heterozygote for common *TSEN54* mutation. (**B**) Coronal image shows extremely small and flattened cerebellar hemispheres. The inferior vermis projects slightly below the hemispheres. Small appendages below the vermis possibly represent tonsils or flocculi, in this case (coronal view, T_1-weighted, 31+5 weeks). Heterozygote for nonsense mutation in *TSEN54* (p.R353GfsX81) plus heterozygote for common *TSEN54* mutation. (**C**) Increased extracerebral space, wide-open sylvian fossa and shallow frontal sulci indicating delay in neocortical maturation (axial T_2-weighted, 31+5 weeks). Heterozygote for nonsense mutation in *TSEN54* (p.R353GfsX81) plus heterozygote for common *TSEN54* mutation.

RARS2 mutations

RARS2 mutations were found in two patients (Table 4). One case (Ex1, Rankin *et al.*, 2010) had progressive microcephaly, hypotonia, no dystonia/dyskinesia, impaired swallowing, seizures and lactic acidemia in the neonatal period. MRI findings were comparable to those published previously by others (Edvardson *et al.*, 2007).

The other case (Sf1) had hypotonia, early lethality (age at death 6 days) and high CSF lactate levels, fitting a pontocerebellar hypoplasia type 6 phenotype. Post-mortem examination of this patient revealed a neuropathological profile that fits a pontocerebellar hypoplasia type 1 phenotype, with loss of spinal anterior horn cells and diffuse gliosis. Furthermore, flat cerebellar folia, loss of Purkinje cells and pontocerebellar hypoplasia were found.

Pontocerebellar hypoplasia type 2 phenotype in the negative mutation group

We compared the group without mutations (63 patients) to the group with mutations, in order to find characteristics common to both groups, as well as atypical features. Using these criteria, only 13 individuals from the no-mutation group retained all the significant characteristics of the common *TSEN54* mutation group (Table 7). However, it must be stated that the clinical profile of the characteristics of these 13 individuals is incomplete.

Unidentified variants

Rare sequence variants were not likely to be pathogenic, since they were synonymous mutations, mutations in introns or untranslated regions, or mild amino acid substitutions (Supplementary Table 1).

Table 7 Inclusion and exclusion criteria in negative mutation group

Inclusion criteria
Jitteriness, clonus
Progressive microcephaly
Dyskinesia/dystonia
Impaired swallowing
Central visual impairment
MRI: Cerebellar hemisphere (score 1)[a]
MRI: Pons (score 1 or 2)[b]
Exclusion criterion
Primary optic atrophy

The above-mentioned characteristics were used as inclusion and exclusion criteria in order to study the negative mutation group more closely. Thirteen patients from the negative mutation group fit the pontocerebellar hypoplasia type 2 phenotype.
a Cerebellar hemisphere score as in Tables 2 and 3.
b Pons score as in Tables 2 and 3.

Discussion

In this study, we investigated a cohort of 169 patients (141 families) clinically diagnosed with pontocerebellar hypoplasia and determined the contribution of five genes known to be involved in pontocerebellar hypoplasia (*TSEN54, TSEN2, TSEN34, RARS2* and *VRK1*). In addition, we sequenced candidate gene *TSEN15* for mutations, since this gene also encodes for a protein-subunit of the TSEN complex. We identified disease-causing mutations in 106 cases, representing a mutation frequency of 62.7%. In 100 patients we identified disease-causing mutations in *TSEN54* (59.2%). Eighty-eight of these patients had a homozygous p.A307S mutation in *TSEN54* (52.1%). Twelve cases had a rare combination of *TSEN54* mutations (7.1%). Three individuals carried mutations in *TSEN2* (1.8%). One patient had a mutation in *TSEN34* (0.6%).

Two patients carried *RARS2* mutations (1.2%). In the genes *TSEN15* and *VRK1* no pathogenic mutations were found. Most alleles with the p.A307S *TSEN54* mutation were from patients of Northern European descent, including cases from the USA and Canada. Exceptional cases were from Israeli, Arab, Turkish and Ibero-American descent.

TSEN54 common mutation: pontocerebellar hypoplasia type 2

The common mutation group fits the pontocerebellar hypoplasia type 2 phenotype (Table 7), in which dyskinesia and/or dystonia and severe microcephaly are the major clinical hallmarks, together with pontocerebellar hypoplasia. The presence of progressive microcephaly and the absence of primary optic atrophy can be considered distinctive features for the common mutation. Epileptic seizures are common in the pontocerebellar hypoplasia type 2 group (81.5%) and the probability of developing these increases with age. In some cases it may be difficult to differentiate between seizures and dyskinetic movements.

Life expectancy is difficult to predict as survival ranges from early postnatal (2.5 weeks) to adult death (one case alive at 31 years). Survival will be prolonged with better care such as tube feeding via percutaneous endoscopic gastrostomy and artificial ventilation.

We show that the pontocerebellar hypoplasia type 2 phenotype is distinct and can be used to guide molecular diagnosis. All cases with the common mutation (100%) have a dragonfly-like phenotype on MRI, therefore MRI is recommended and helps in early diagnosis.

Severe *TSEN54* mutations: pontocerebellar hypoplasia type 4

Nine patients were considered to have a combination of missense and nonsense mutations in *TSEN54*, leading to more severe symptoms (Table 4) and early death (median age 12 days). These patients fit the pontocerebellar hypoplasia type 4 subtype.

MRI analysis of the *TSEN54* severe mutation group deviates in some regards from the common mutation group: analysis of seven cases reveals pericerebral CSF accumulation, persistently wide midline cava, delayed neocortical maturation and more severe involvement of the vermis (Fig. 6A–C). MRI analysis is therefore essential for diagnosis of pontocerebellar hypoplasia type 4.

RARS2 mutations causing a pontocerebellar hypoplasia type 1-like phenotype in one case

In one pontocerebellar hypoplasia type 1 case (confirmed by autopsy) we identified disease-causing mutations in *RARS2*. These mutations were not previously associated with pontocerebellar hypoplasia type 1, but *RARS2* mutations are associated with pontocerebellar hypoplasia type 6, featuring mild mitochondrial chain defects, hypotonia, progressive microcephaly and elevated CSF lactate levels. High CSF lactate is not reported in patients with a *TSEN* mutation. Autopsy was not performed on the previously published patients with pontocerebellar hypoplasia type 6 (Edvardson *et al.*, 2007; Rankin *et al.*, 2010), and therefore a relationship between *RARS2* and pontocerebellar hypoplasia type 1 phenotype cannot be excluded until further cases have been studied.

TSEN2, *TSEN34* and rare *TSEN54* missense mutations

In seven cases of pontocerebellar hypoplasia (Le1, Pa1 II.1, Pa1 II.3, Hg1, Dh1, Vi5, Lj1) we identified rare missense mutations in *TSEN34* and *TSEN54*, and missense and splice site mutations in *TSEN2*. They fit the pontocerebellar hypoplasia type 2 phenotype; however Patient Dh1 has a mild clinical phenotype compared to the common *TSEN54* mutation group. Since this group of patients is small in number, more individuals will be necessary to draw a specific phenotypical profile.

Unsolved cases

The mutation-negative group is phenotypically heterogeneous in contrast to the common mutation group. Only 13 of the 63 cases fulfilled the common mutation characteristics (criteria in Table 7). Information on some of these characteristics is missing in these 13 individuals, showing that sufficient clinical information and MRI are essential for confident clinical diagnosis and genetic analysis. Untranslated regions, introns or mutations in other genes may underlie the pathogenesis in these cases.

All patients referred by clinical geneticists or neurologists with the clinical or radiological diagnosis pontocerebellar hypoplasia were entered into this study reflecting common clinical practice. Taking these non-strict selection criteria into account, it remains possible that congenital glycosylation disorders, mitochondrial disorders or other diseases underlie the neurological findings in some of the unsolved cases (Denecke *et al.*, 2005; Scaglia *et al.*, 2005; van de Kamp *et al.*, 2007). In case of sensorineural hearing loss and a simplified gyral pattern, X-linked calcium/calmodulin-dependent serine protein kinase mutations should be considered (Najm *et al.*, 2008). One should consider progressive encephalopathy, oedema, hypsarrhythmia and optic atrophy syndrome when a case presents with these symptoms (Somer, 1993). Stricter inclusion criteria might increase the percentage of mutation-positive cases.

Furthermore, the unsolved cases suffer from a higher rate of premature births (34.5%) than the common mutation group (17.5%). In these cases, the severe prematurity (<32 weeks) may be associated with pontocerebellar disruption, developmental delay and the occurrence of seizures (Messerschmidt *et al.*, 2008). Four (13.8%) of the unsolved cases were severely premature (born at 27 and 29 weeks, two others were born at 32 weeks). In the common *TSEN54* mutation group there were three cases (4.8%) with severe prematurity (one case at 29 weeks and twins at 30 4/7 weeks, Graham *et al.*, 2010).

Unclassified variants

In three cases, we cannot exclude that the missense mutations found on one allele are not associated with pontocerebellar hypoplasia (*TSEN54* p.N347Y, p.R374C and *TSEN34* p.R279C, Supplementary Table 1). Since it is not possible to detect large deletions by direct sequencing, we cannot rule out the possibility that these variants are pathogenic in combination with a deletion on the opposite allele.

Disease mechanism

It is difficult to define a common pathway in the different genes associated with pontocerebellar hypoplasia. However all genes, apart from *VRK1*, play a role in protein synthesis and transfer RNA processing in particular. *RARS2* encodes for mitochondrial arginyl transfer RNA synthetase, which charges arginine to specific transfer RNAs during protein synthesis. *TSEN2, TSEN34, TSEN54* and *TSEN15* encode for the transfer RNA splicing endonuclease, which plays a role in intronic transfer RNA splicing (http://lowelab.ucsc.edu/GtRNAdb/) (Paushkin *et al.*, 2004). Alternative functions of these proteins might play a role in a common pathway responsible for pontocerebellar hypoplasia (Paushkin *et al.*, 2004). Other neurodegenerative diseases such as Charcot-Marie-Tooth disease and leukoencephalopathy with brainstem and spinal cord involvement and elevated lactate, are also associated with mutations in transfer RNA synthetase genes (Seburn *et al.*, 2006; Scheper *et al.*, 2007; Antonellis and Green, 2008; Park *et al.*, 2008). Patients with nonsense mutations in *TSEN54* show a more severe phenotype, which suggests that loss of function of the TSEN54 protein is the underlying disease mechanism. Further research is required to investigate why mutations in transfer RNA processing genes lead to pontocerebellar hypoplasia.

Conclusion

Here we present evidence that the common homozygous mutation in *TSEN54* can be predicted reliably from the pontocerebellar hypoplasia type 2 phenotype. There is a strong association with flat dragonfly-like cerebellar hemispheres, a flat pons, dyskinesia and/or dystonia, neonatal irritability, central visual impairment, the absence of optic atrophy and severe cognitive and motor impairment. More severe *TSEN54* mutations are associated with early postnatal death, contractures, polyhydramnios and ventilatory problems. MRI analysis reveals severe cerebral immaturity of the cortex, which assists in establishing a diagnosis of pontocerebellar hypoplasia type 4. Our data provide further evidence that pontocerebellar hypoplasia type 2 and 4 result from a common spectrum of mutations, mainly affecting *TSEN54*. The homogeneity of the phenotype, both from a clinical perspective and by neuroimaging, correlates strongly with the genotype and can facilitate early diagnosis and assist in molecular genetic testing. A clinical and/or neuroradiological pontocerebellar hypoplasia type 2 profile is shared with 13 unsolved cases, suggesting the involvement of other unidentified genes or the involvement of non-coding regions in the *TSEN* or *RARS2* genes in pontocerebellar hypoplasia. In addition, we show that pontocerebellar hypoplasia type 1 together with elevated CSF lactate may be caused by *RARS2* mutations. Together these data enhance the clinical description of pontocerebellar hypoplasia and will assist with the neuroradiological and genetic diagnosis of pontocerebellar hypoplasia.

Acknowledgements

We thank M.B. de Wissel-Dijns for technical assistance. We are grateful to Prof. J.M.B.V. de Jong, Prof. M. de Visser and K. Ritz for critical reading of the manuscript. We acknowledge the contribution of subject's data and DNA samples by S. Shallat, Peoria Medical Centre, Peoria, IL, USA; W. Deppe, Klinik Bavaria, Kreischa; H. Brunner, Radboud University Nijmegen; Miranda Splitt, The Institute For Human Genetics, Newcastle upon Tyne, UK; Helen Cox, West Midlands Regional Clinical Genetics Service, Birmingham Womens Hospital, Edgbaston, Birmingham, UK; M. Steinlin, University Hospital, Bern; A. Böhring, Westfälische Wilhelms-Universität, Münster, Germany; S. Gebre-Medhin, Universal Hospital, Lund, Sweden; K. Schoppmeyer, Universitätsklinikum Leipzig, Germany; D. Rowitch, Departments of Paediatrics, Neurosurgery and Howard Hughes Medical Institute, UCSF, San Francisco, USA; M. Koch, Neuropaediaty, Vestische Kinder-Jugendklinik, Datteln.

Funding

Financial support was kindly provided by the Hersenstichting Nederland KS2009(1)-81 and the AMC Graduate School, University of Amsterdam. Y.J.C. acknowledges the Manchester NIHR Biomedical Research Centre.

Supplementary material

Supplementary material is available at *Brain* online.

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Appendix 1

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