Journal of the Neurological Sciences 344 (2014) 37–42

Contents lists available at ScienceDirect

Journal of the Neurological Sciences

journal homepage: www.elsevier.com/locate/jns

Clinical and genetic analysis in alternating hemiplegia of childhood: Ten new patients from Southern Europe

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ARTICLE INFO

Article history:
Received 26 March 2014
Received in revised form 4 June 2014
Accepted 9 June 2014
Available online 17 June 2014

Keywords:
Alternating hemiplegia of childhood
ATP1A3
CACNA1A
Genetics
Ketogenic diet
Neurodevelopmental syndrome

ABSTRACT

Alternating hemiplegia of childhood (AHC) is a rare neurodevelopmental disorder featuring attacks of hemiplegia and other paroxysmal and non-paroxysmal manifestations leading to progressive neurological impairment. De novo mutations in *ATP1A3* have been identified in up to 80% of patients. AHC is also associated with rare mutations in other genes involved in episodic neurological disorders.

We sought to find mutations in *ATP1A3*, *CACNA1A*, *ATP1A2*, *SCN1A* and *SLC2A1* in a cohort of ten unrelated patients from Spain and Greece.

All patients fulfilled AHC diagnostic criteria. All five genes were amplified by PCR and Sanger sequenced. Copy number variation (CNV) analysis of *SLC2A1* and *CACNA1A* was performed using two different approaches.

We identified three previously described heterozygous missense *ATP1A3* mutations (p.Asp801Asn, p.Glu815Lys and p.Gly947Arg) in five patients. No disease-causing mutations were found in the remaining genes. All mutations occurred *de novo*; carriers presented on average earlier than non-carriers. Intellectual disability was more severe with the p.Glu815Lys variant. A p.Gly947Arg carrier harbored a maternally-inherited *CACNA1A* p.Ala454Thr variant. Of note, three of our patients exhibited remarkable clinical responses to the ketogenic diet.

We confirmed *ATP1A3* mutations in half of our patients. Further AHC genetic studies will need to investigate large rearrangements in *ATP1A3* or consider greater genetic heterogeneity than previously suspected.

1. Introduction

Alternating hemiplegia of childhood (AHC) is a complex and rare neurodevelopmental syndrome that was first described by Verret and Steele in 1971 [1]. It is characterized by (i) onset of paroxysmal events before 18 months of age, (ii) repeated periods of hemiplegia involving either side of the body lasting from a few minutes to several days caused by various factors including emotional triggers, head trauma and fatigue, (iii) episodes of bilateral hemiplegia or quadriplegia of varying intensity, (iv) other paroxysmal manifestations including tonic and dystonic episodes, ocular abnormal movements (nystagmus, strabismus) and/or autonomic disturbances occurring during hemiplegic bouts or in isolation, (v) disappearance of all abnormalities by sleep, with probable recurrence of long-lasting bouts after waking, and (vi) nonparoxysmal neurological abnormalities including developmental delay, choreoathetosis, dystonia and/or ataxia [2,3].

Analysis of whole exome sequencing in 16 proband-parent trios and whole genome sequencing in another two led to establish AHC as a genetic disorder caused by mutations in *ATP1A3*, encoding the neuronal α_3-subunit of the Na^+/K^+-ATPase pump [4–6]. Subsequent molecular analysis in 143 additional AHC patients revealed the presence of mutations in 112 of them [4–8]; the negative results in approximately 20% of patients may indicate some degree of genetic heterogeneity in AHC. In fact, some reports have linked AHC, or a very similar phenotype, to mutations in three genes encoding ionic channels or solute carriers expressed in the central nervous system: *CACNA1A* [9], *ATP1A2* [10,11] and *SLC2A1* [12].

In the present study, we sought to determine whether mutations in *ATP1A3* or in any of the three genes involved in familial hemiplegic migraine (FHM), *CACNA1A*, *ATP1A2* and *SCN1A* or in glucose transporter

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http://dx.doi.org/10.1016/j.jns.2014.06.014
0022-510X/

type 1 deficiency syndrome (GLUT1DS), *SLC2A1*, were linked to AHC in a cohort of 10 unrelated patients from Spain and Greece.

2. Subjects

Ten sporadic AHC patients who clinically fulfilled the previously described criteria for the disorder [2,3] were recruited by neurologists at four Spanish or Greek centers. After obtaining informed consent from all patient parents or custodians, blood samples were collected and genomic DNA was extracted following standard procedures [13]. The study was approved by the local Ethics Committee at Vall d'Hebron University Hospital, Barcelona.

3. Methods

3.1. Mutation screening

All promoters, exons and flanking intronic regions of *ATP1A3*, *CACNA1A*, *ATP1A2* and *SLC2A1* genes and the five FHM-associated *SCN1A* exons (number 6, 17, 23, 24 and 26) and flanking intronic regions were amplified by PCR in all the patients (details available upon request). Purified PCR products were sequenced using the BigDye Terminator cycle sequencing kit v3.1 and the automated sequencer ABI PRISM 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). All mutations were assessed by bidirectional sequencing. Inheritance of mutations was determined after sequencing the parents of all mutation carriers.

Mutation nomenclature follows HGVS guidelines (www.hgvs.org/mutnomen) and refers to the *ATP1A3* cDNA sequence NM_152296.4 (protein sequence NP_689509.1) and to the *CACNA1A* cDNA sequence NM_023035.2 (protein sequence NP_075461.2) with +1 corresponding to A of the ATG translation initiation codons.

3.2. Copy number variant analysis

Copy number variation (CNV) studies for *SLC2A1* and *CACNA1A* were performed. For *SLC2A1* we analyzed all exons with the Multiplex Ligation-Dependent Probe Amplification (MLPA) assay by using the SALSA MLPA kit P138 for *SLC2A1* (MRC-Holland, Amsterdam, the Netherlands). For *CACNA1A* we used two complementary approaches to maximize gene coverage: MLPA and Quantitative Multiplex PCR of Short Fluorescent Fragments (QMPSF). For the MLPA assay we used the SALSA MLPA kit P279-A2 for *CACNA1A* (MRC-Holland, Amsterdam, the Netherlands) and for QMPSF we used four sets of primer pairs that covered 16 additional exons not included in the MLPA kit (experimental details and primer sequences available upon request).

4. Results

4.1. Clinical data

The main clinical features of the ten patients (5 females) are summarized in Table 1.

Briefly, all patients were from Southern Europe, seven from Spain and three from Greece. The age of onset varied from 0 to 18 months; present age is comprised between 6 and 36 years. Attacks lasted minutes to days with a daily to every two months frequency and featured typical motor signs including unilateral or bilateral paresis, hypotonia, dystonia, rigidity, ataxia, nystagmus or other abnormal eye movements, dysphagia, hand posturing and postictal drowsiness. Other frequently reported paroxysmal events included epileptic seizures, cyclic vomiting and migraine with aura. Over a 6–16 year follow-up period, 9/10 patients have developed different degrees of intellectual deterioration, mostly moderate to severe, and other signs of chronic, progressive neurological dysfunction, such as ataxia, dysarthria, spasticity, hypotonia, hypertonia, dyskinesia, tremor and pyramidal signs. Microcephaly occurred in only one patient.

All patients had MRI studies, which were all normal except for patient 6 who displayed subtle bihemispheric white matter lesions. All patients showed no abnormalities on serial EEGs.

None of the patients had familial history of AHC, however six had familial history of migraine, one of a non-classified neurodegenerative disorder, one of vertigo and one of epilepsy.

Used treatments included prophylaxis with flunarizine, which often provided some degree of improvement in severity and frequency of attacks, or topiramate. Interestingly, in three patients the number and severity of attacks were markedly reduced upon institution of the ketogenic diet (KD). Patient 1 was treated for one year with KD, at age 11. She had been on flunarizine since age 3 with no clear benefit. The frequency of her dystonic attacks while on KD decreased from one per week to one every three weeks, approximately. A more dramatic improvement was recorded in her behavioral status. Both her psychologist and caregivers described frank improvement in her school performance and resolution of mood swings and sociability problems. Because of cost-effectiveness issues the family decided to stop the treatment. Patients 4 and 5 were treated with flunarizine since the disease started with poor response. Benzodiazepines were used to take them quickly to sleep and shorten the attacks: in patient 5 rectal diazepam reduced the duration of her attacks from 2 to 3 days to 4–5 h, but frequency (1–2 a week) remained unchanged; in the case of patient 4 clonazepan in his oral mucosa reduced the attacks to 3–4 h instead of 1–2 days, but their number did also not decrease. In both of them KD brought about a cessation of the hemiplegic attacks. In addition, and according to teachers and caregivers, there were improvements in their motor clumsiness, mood. attention and, though not quantified, global cognitive functions. Of note, patient 5, a 6 year-old girl, has suffered no further attacks since she was put on the diet at age 4.

4.2. Mutation screening

The extensive sequencing of the *ATP1A3* gene in ten subjects with AHC allowed the identification of three previously reported changes in five unrelated patients. We identified a G-to-A transition at cDNA position c.2401, resulting in the substitution of an aspartic acid for an asparagine at residue 801 (p.Asp801Asn), in patient 1; a G-to-A transition at cDNA position c.2443, resulting in the substitution of a glutamic acid for a lysine at residue 815 (p.Glu815Lys), in patient 2; and a G-to-A transition at cDNA position c.2839, resulting in the substitution of a glycine for an arginine at residue 947 (p.Gly947Arg), in patients 3, 4 and 5 (Fig. 1). All mutations were heterozygous and were confirmed to be *de novo*, except in patient 1 whose parental DNA was not available. However, the variant found in this patient has been previously associated with AHC in the literature.

A distinctive clinical feature of *ATP1A3*-positive vs *ATP1A3*-negative patients was an earlier onset of symptoms in the former group (average age of onset: 4.8 vs 13.4 mo).

Moreover, direct sequencing of *CACNA1A* gene revealed a heterozygous G-to-A transition at cDNA position 1360 (c.1360G>A) in patient 5, who also bore an *ATP1A3* change, and in her asymptomatic mother. This mutation results in the substitution of alanine for threonine at residue 454 (p.Ala454Thr) and is assessed as probably damaging by the prediction tool PolyPhen-2 v2.2.2 (score = 1). All mutations are listed in Table 2.

No mutations in *ATP1A2*, *SCN1A* and *SLC2A1* genes nor copy number variants in *SLC2A1* or *CACNA1A* were found in the ten patients screened.

5. Discussion

We have identified three mutations in the *ATP1A3* gene in five out of ten AHC patients from Spain and Greece. *ATP1A3* encodes the alpha-3 catalytic subunit of te Na^+/K^+ ATPase pump. Na^+/K^+ ATPases maintain

Table 1
Clinical features of 10 patients with alternating hemiplegia of childhood. M: male; F: female; mo: months; MO: migraine without aura; MA: migraine with aura; HM: hemiplegic migraine; KD: ketogenic diet; ID: intellectual disability; –: no information. Shading indicates *ATP1A3* mutation carrier.

Patient	Origin	Present age (years) and gender	Age at onset (mo)	Attack description	Body part involvement	Duration	Frequency	Other paroxysmal events	Interictal exam	Other	Family history	Treatment (response)
	Greece	16 F	6	Paresis Dystonia	Unilateral	Minutes–to–hours	Daily–to–weekly	–	Mild ID Dysarthria Mild–moderate spasticity Ataxia	Normal MRI, EEG, SPECT	No	Multiple antiepileptic drugs and flunarizine (no effect) KD (improved)
2	Greece	7 M	4	Flaccid paresis Ataxia	Unilateral	1–3 days	1–2/week	Dyskinesia	Severe ID and motor involvement	Normal MRI, EEG	No	Flunarizine (improved)
3	Spain	16 F	4	Flaccid paresis	Bilateral	2–7 days	2/month	Rigidity episodes	Mild ID Cerebellar ataxia	Normal MRI C677T mutation in MTHFR	MO	–
4	Spain	37 M	3	Ataxia Flaccid paresis Dystonia	Unilateral	Hours/days	2–4/month	Autonomic dysfunction	Moderate ID	Normal MRI, arteriography and EEG	No	Flunarizine (no effect) KD (improved) Diazepam (shortened attacks)
5	Spain	6 F	0	Nystagmus Chorea Dystonia Flaccid paresis	Unilateral	1–12 days	1/month	–	Mild ID Squint	Normal MRI, EEG Abnormal SPECT	MO	Flunarizine & topiramate (no effect) KD (attacks abated)
6	Spain	13 M	15	Flaccid paresis “drowsiness postictally”	Unilateral	1h–1 week	6/year	Epilepsy	Severe ID	White matter hyperintensities on MRI	No	Flunarizine (improved)
7	Spain	8 F	13	Hypotonia Nystagmus Dysphagia Hand posturing	Unilateral Bilateral	2–6 days	1/month	Nystagmus	Clumsiness Normal cognition	Normal MRI, EEG Abnormal ictal SPECT	MO	Flunarizine (improved)
8	Greece	9 M	18	Ataxia Flaccid paresis	Unilateral	Minutes–to–days	2/week	–	Developmental delay Tremor Ataxia	Normal MRI, EEG	MO Vertigo Paresthesia	Topiramate (improved)
9	Spain	16 F	3	Flaccid paresis	Unilateral	1–2 days	1/month	Epilepsy Cyclic vomiting	Severe ID Spasticity	Normal MRI, EEG	MO Epilepsy	–
10	Spain	12 M	18	Nystagmus Chorea Dystonia	Unilateral	Minutes–to–days	1–2/month	Epilepsy MA Dystonia Myoclonus	Severe ID	Normal MRI, EEG	MO HM	Diazepam (shortened attacks)

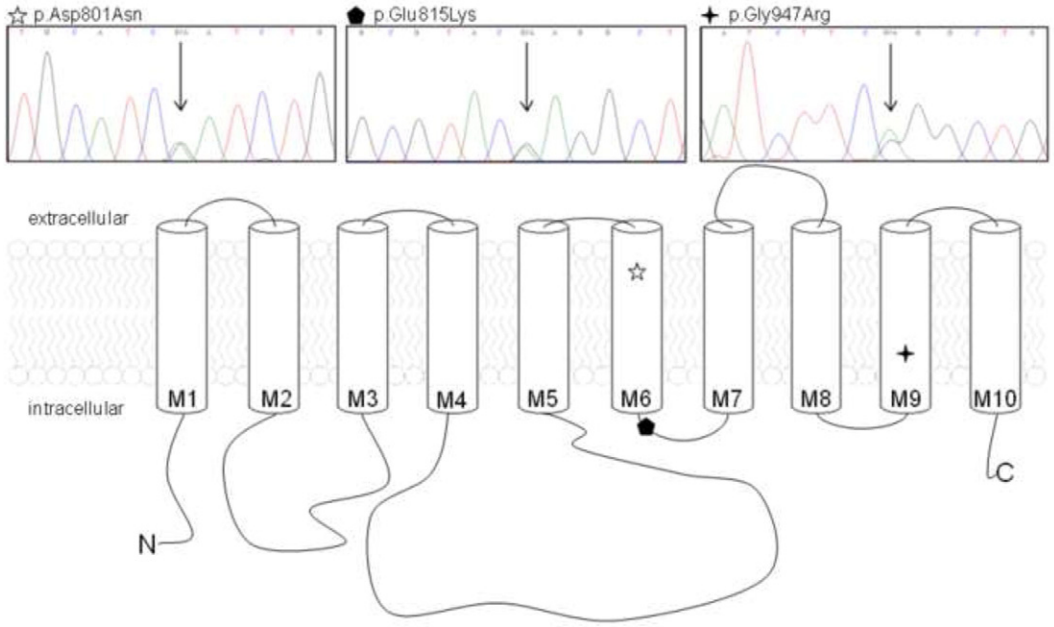

Fig. 1. Electropherograms of *ATP1A3* changes found in AHC patients and diagram of the ATP1A3 protein indicating the positions of the mutations. Three *ATP1A3* mutations were found in five AHC patients. Mutations p.Asp801Asn and p.Gly947Arg are located within transmembrane domains M6 and M9, respectively, and p.Glu815Lys within cytoplasmic loop M6–7.

cationic gradients essential for a number of cell membrane functions, including muscle and nerve electrical excitability [13]. The ATPase alpha-3 isoform, in turn, appears to play a critical role during high-frequency activity, when intracellular Na^+ increases. Mutations in *ATP1A3*, have been described to cause at least two phenotypes: AHC and rapid-onset dystonia-parkinsonism (RODP) (DYT12), a movement disorder characterized by abrupt onset of dystonia, usually accompanied by signs of parkinsonism. A recent study proposes that the two disorders lie at both ends of a continuous phenotypic spectrum of *ATP1A3*-related disorders [15]. It was hypothesized that inability to maintain the high demand for ion transport during and after stressful events may relate to the development of abnormal movements in ROPD [14]. This might be also the case in the more severe AHC phenotype, where even small stressful events may bring about more frequent and severe episodes, including status epilepticus [8]. In fact, presence of epilepsy may reflect a more profound pump dysfunction and in our series it was associated with worse global outcome.

There is evidence that the three identified amino acid substitutions are disease-causing: all are missense, have been previously described in several AHC patients and appeared *de novo*, as shown in all available trios. The mutations identified in our cohort are also the most frequent AHC-causing mutations in the five previous *ATP1A3*-screened case series, where p.Asp801Asn was the most frequent, followed by p.Glu815Lys and p.Gly947Arg [4–8]. In our series, p.Gly947Arg was the most frequently encountered mutation. All of these changes are G>A transitions located within hypermutable CpG dinucleotide sequences and have been only described as *de novo* mutations.

Recently, a Japanese study on 33 AHC cases attempted to establish genotype–phenotype correlations by grouping their case series according to the following *ATP1A3* mutation types: p.Asp801Asn, p.Glu815Lys or other mutations [8]. It was suggested that the p.Glu815Lys group had a more severe clinical course, while the p.Asp801Asn group resulted in a moderate form of AHC. Our results, despite the smaller sample size, may concur with these findings, since patients with the p.Gly947Arg or the p.Asp801Asn variants all had mild to moderate intellectual disability whereas the patient bearing the p.Glu815Lys variant had a severe and global psychomotor involvement. Conversely, patients with no mutation in *ATP1A3* also displayed variable degrees of neurological impairment, though they tended to present later in life.

Compared to ours, previous genetic studies in AHC showed a higher incidence of *ATP1A3* mutations. Although we have not ruled out the presence of *ATP1A3* CNVs in our patients, data derived from the functional analyses of *ATP1A3* mutations suggest that such CNVs would be more apt to produce a DYT12 phenotype, since DYT12-causing mutations reduce protein expression, whereas AHC-causing mutations seem to modulate pump activity [5,14]. Also, the possibility of additional AHC loci remains open, particularly considering the specific geographic origin of the present cohort.

Prior to the description of *ATP1A3* as the major genetic cause of AHC, genetic screenings in smaller AHC cohorts, or single case reports, identified mutations in three genes encoding ion-channels, i.e. *ATP1A2* [10,11], *CACNA1A* [9] or the solute carriers *SLC2A1* [12] and *SLC1A3* [16]. The proteins encoded by three of these genes play important roles at glutamatergic synapses: *CACNA1A* encodes a presynaptic neuron

Table 2
Mutations found in five out of 10 patients with alternating hemiplegia of childhood.

Patient	Gene	Mutation				
		Exon	cDNA level	Protein level	Inheritance	Frequency in AHC patients screened for this gene (see refs. [4–8])
1	*ATP1A3*	17	c.2401G>A	p.Asp801Asn	Parental DNA unavailable	48%
2	*ATP1A3*	18	c.2443G>A	p.Glu815Lys	De novo	33%
3	*ATP1A3*	21	c.2839G>A	p.Gly947Arg	De novo	8%
4	*ATP1A3*	21	c.2839G>A	p.Gly947Arg	De novo	8%
5	*ATP1A3*	21	c.2839G>A	p.Gly947Arg	De novo	8%
	CACNA1A	11	c.1360G>A	p.Ala454Thr	Inherited from asymptomatic mother	17%

calcium channel involved in glutamate neurosecretion, while *ATP1A2* encodes the astrocytic ATPase isoform α_2 involved in glutamate reuptake; *SLC1A3*, in turn, encodes the glial glutamate and aspartate transporter EAAT1. Because of these previous findings and the existing clinical overlap between severe forms of FHM [17] and atypical AHC, we performed sequence analysis of the three known FHM genes and of *SLC2A1*, which encodes the glucose transporter at the blood–brain barrier. Mutations in *SLC2A1* are the cause of GLUT1DS, a syndrome that shows wide phenotypic variability and shares many clinical signs with AHC, such as delayed development, episodic eye movements, transient abnormal involuntary movements – including hemiparesis – and epilepsy. Mutational analysis of these four genes did not reveal the genetic cause of the disease in any of the patients that were negative in the *ATP1A3* screen. This agrees with a recent Italian study investigating *SLC2A1* mutations in AHC [18]. However, we identified the p.Ala454Thr variant in *CACNA1A* in patient 5 and her asymptomatic mother. This mutation was first considered a polymorphic variant with a frequency of 0.02 in the control population of a genetic screen performed in FHM and episodic ataxia patients [19]. It was later associated with early-onset progressive ataxia [20]. More recently, we found this mutation in two subjects displaying the milder phenotype in a family segregating both FHM and migraine with aura [21]. The functional in vitro analysis concluded that this mutation reduced the secretion efficiency of the channel, which prompted us to consider the p.Ala454Thr mutation as a negative modulator of the aura severity. Our patient 5 is also carrying an *ATP1A3* mutation. A possible relationship between CACNA1A and ATP1A3 proteins has only been considered in a study where presynaptic Ca^{2+} buffers were shown to control the strength of a fast post-tetanic hyperpolarization mediated by the ATP1A3 pump [22]. This led us to speculate that the consequences of the *ATP1A3* mutation in patient 5 could be modulated by the found *CACNA1A* variant and result in a milder AHC phenotype. It is conceivable that the many genes regulating membrane excitability are liable to act as each other's modifiers in paroxysmal neurological phenotypes and that variable expression in these dominantly inherited disorders may relate to epistatic or other types of gene–gene interaction.

A unique feature of three of our patients was the clinical response to KD institution, particularly concerning the paroxysmal symptoms. This concurs with two recent observations of KD-induced amelioration of paroxysmal signs in two AHC patients carrying mutations in *ATP1A3*, both initially diagnosed with GLUT1DS and one of them effectively harboring a *SLC2A1* rare variant [23,24]. At present there is no clear rationale for the use of KD therapy in AHC. Use in our patients was empirical and occurred before molecular diagnosis was known. Institution of the diet was decided in patients with very frequent attacks and lack of response to other treatments, and was based on previous observations of an AHC-like phenotype in GLUT1DS [12] and the finding of interictal abnormal cerebral glucose metabolism in the frontal lobes, ipsilateral putamina and cerebellum in AHC Japanese patients, as detected by means of FDG-PET studies [25]. Whatever the mechanism, all three patients where KD was tried underwent a substantial reduction of attacks and a long-lasting resolution in one (patient 5), only to recur very recently in association with intense emotional stress.

Our results confirm *ATP1A3* mutations as a common cause of AHC at both ends of the Mediterranean area, but also raise the issue of the existence of genetic heterogeneity. Studies focused on AHC patients who are negative for mutations in *ATP1A3*, including data mining of their existing massive sequencing results will hopefully identify novel genes or deep-intronic sequence variants associated with this devastating disorder.

Note added in proof

During the processing of this article, we performed a MLPA analysis, using the SALSA MLPA P059 Dystonia probemix (MRC Holland, Amsterdam, The Netherlands), of samples from patients 6-10. No *ATP1A3* CNVs were detected.

Conflict of interest

The authors have no conflict of interest to declare.

Acknowledgments

We thank the patients and their families for accepting to participate in this study. We acknowledge the valuable contribution of referring physicians, M.T. García-Silva and M.R. Domingo. M.V-P is supported by Fundació Institut de Recerca Vall d'Hebron and C.S. by MINECO (Spain). This work is supported by Agència de Gestió d'Ajuts Universitaris i de Recerca (Catalunya, Spain), grants SGR 2009/0078 and SGR 2009/0971.

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