A novel mutation in the ATP1A2 gene causes alternating hemiplegia of childhood

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Alternating hemiplegia of childhood (AHC, MIM 104290) is a rare syndrome, characterised by early onset of episodic hemi- or quadriplegia lasting minutes to days. This disorder, first reported by Verret and Steel in 1971,1 has historically been thought to represent a migraine equivalent or an unusual form of epilepsy or a movement disorder,2 as it typically presents with complex and variable clinical features.

In most patients, the earliest manifestations clearly related to AHC are tonic–dystonic attacks and paroxysmal nystagmus associated with autonomic changes and paroxysmal dyspnoea and usually appear between 3 and 6 months of age. The hemiplegic episodes develop before 18 months of age lasting anywhere from minutes to days at a time and involving either side of the body or shifting from one side to the other during the same episode with a period of bilateral weakness when the second side becomes involved.3

Some attacks are characterised by a bilateral involvement, which is apparent from the beginning, and do not follow an hemiplegic episode,2 which determines extreme hypotonia of the whole body with inability to move and a level of consciousness markedly depressed.

A characteristic feature of AHC is the disappearance of all abnormalities when the child falls asleep.4 With increasing age, hemiplegic episodes follow a general pattern of initially increasing frequency and duration, followed by a plateau, and finally by a decrease in the number and duration of attacks. Analogously, the paroxysmal manifestations associated with hemiplegia tend to decrease in frequency and intensity with time and usually disappear after 5–7 years. Development before the onset of hemiplegic episodes may be normal or delayed.

Epileptic seizures are reported in a variable percentage of sporadic patients.3–5 An association with migraine has also been noticed since the earliest description.1 Indeed, for some time, the international classification of headaches (IHC) has included AHC as a familial hemiplegic migraine, FHM,6 mostly due to the clinical similarity of the attacks and the paroxysmal nature of both disorders. Some of the aspects of AHC, however, are clearly distinct from FHM, including choreoathetosis, dystonic posturing, and a progressive course associated with mental deterioration.7 Concerning the possible genetic origin of the disease, only a few familial cases have been described so far, providing evidence to support an autosomal dominant genetic mechanism of transmission.8,9

In the first AHC family described, a balanced reciprocal translocation was detected: 46,XY, t(3;9)(p26;q34), thus indicating the first two genomic regions possibly associated with the disease.4 Subsequently, mitochondrial abnormalities identified in a few sporadic AHC patients suggested the hypothesis, which was however never clearly confirmed, of a mitochondrial dysfunction underlying the pathogenesis of the disease.10

In the present study, we fully describe a new AHC family from a Greek island with four affected members in two generations, the mother and three out of four children. All patients share a normal karyotype. Due to the partial clinical overlap of familial hemiplegic migraine (FHM) with AHC, we screened the ATP1A2 gene coding for the α2 subunit of the Na,K pump, associated with FHM type 2.

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We found a novel heterozygous mutation segregating with the disease and causing a threonine to asparagine replacement (T378N). This missense mutation localises to the ATPases phosphorylation site of the hydrolyase domain. The affected residue is highly conserved in all the known α subunits of the Na,K and Na,H pumps from vertebrates to invertebrates. Functional data suggest that loss of function of the mutated ATP1A2 isoform is involved in generating the disease phenotype. This is the first mutation associated with AHC identified so far.

Key points

- Alternating hemiplegia of childhood (AHC, MIM 104290) is a rare syndrome, characterised by early onset of episodic hemi- or quadriplegia lasting minutes to days. The majority of patients are sporadic. Only a few familial cases are reported in the literature.
- Here we describe a new familial case from a Greek island with four affected members in two generations, the mother and three out of four children. All patients share a normal karyotype. Due to the partial clinical overlap of familial hemiplegic migraine (FHM) with AHC, we screened the ATP1A2 gene coding for the α2 subunit of the Na,K pump, associated with FHM type 2.
- We found a novel heterozygous mutation segregating with the disease and causing a threonine to asparagine replacement (T378N). This missense mutation localises to the ATPases phosphorylation site of the hydrolyase domain. The affected residue is highly conserved in all the known α subunits of the Na,K and Na,H pumps from vertebrates to invertebrates. Functional data suggest that loss of function of the mutated ATP1A2 isoform is involved in generating the disease phenotype. This is the first mutation associated with AHC identified so far.

Abbreviations: AHC, alternating hemiplegia of childhood; BFIC, benign familial infantile convulsions; CK, creatin kinase; CT, computed tomography; EEG, electroencephalography; FHM, familial hemiplegic migraine; IHC, international classification of headaches; MRI, magnetic resonance imaging
plasma membrane Na,K pump, a heteromeric protein essential for all mammalian cells that consumes ATP to actively transport Na\(^+\) out and K\(^+\) into the cell. Mutations in ATP1A2 are involved in generating familial hemiplegic migraine type II disease (FHM2) either isolated\(^{11}\) or in association with benign infantile convulsions (BFIC).\(^{12}\) The new mutation identified in our AHC family causes an amino acid change (T378N) falling in the protein hydrolase domain within the consensus site for phosphorylation of the Na,K ATPases. Functional data indicate that this mutation has a loss of function effect although the protein results in the correct plasma membrane location.

This mutation represents the first molecular change associated with the AHC syndrome.

**METHODS**

Case presentation

The pedigree of the AHC family represented in fig 1 shows four affected members in two generations, the mother (II-4) and three out of four children (III-1, III-3, III-4). The second son (individual III-2) is a healthy boy with normal neuropsychological and neuromotor development. The family has been ascertained by two of the authors (NB and KN). The clinical features of the affected members are quite different suggesting high phenotype variability within this family. A blood sample from individual I-3 (deceased) was not available. However, all members of the family describe him as a healthy man throughout his life with no signs or symptoms related to the disease.

**Individual II-4**

The subject is a 38 year old Caucasian woman, delivered at term after a trouble-free pregnancy. She presented some delay in acquiring language skills, but apparently experienced no other problems in achieving developmental milestones. At the age of 2 years, she presented the first attack of alternating hemiplegia; the episode lasted 24 h and was apparently evoked by a head trauma secondary to a fall from the stairs. During the attack, the hemiplegia shifted sides, and during the transition she became tetraparetic and unable to eat or talk. Sleep was associated with resolution of the attack. The resolution persisted for 15–20 min after awakening and was followed by recurrence of the symptoms during wakefulness. Because of this attack, she was hospitalised for 20 days, but a complete neuroradiological examination was never performed. After this first major episode, she suffered only mild hemiplegic attacks, occurring sporadically mostly in adulthood, during pregnancy, and also later, the last having occurred at 38 years of age. No autonomic disturbances were referred during or between hemiplegic attacks. In addition, in the years following the first episode, progressive cognitive deterioration was referred. She had no clinical history of seizures or headache and there were no familial cases of migraine. At the last neurological examination (performed at 42 years of age), choreoathetoid movements and horizontal nystagmus were present with mild gait ataxia. Mild spasticity was noticed and a slight left-side prevalence of tendon reflexes (2\(^+\)); plantar responses were bilaterally flexor. The patient shows mild dysmetria and intention tremor at cerebellar functional tests. Cranial nerve examination was normal as well as position and sensations. At the time of hospitalisation and AHC diagnosis of the first affected son (individual III-1), muscle biopsy was performed and was normal.

At 38 years of age, she was mildly mentally retarded with a persistent disability characterised by difficulty in understanding and performing even simple orders, a limited fund of knowledge, literal interpretation, and lack of understanding of proverbs. However, a complete formal neuropsychological evaluation was never carried out.

**Subject III-1**

The subject is a 12 year boy, born at full term after an uneventful pregnancy and normal delivery. There was no
neonatal distress. Delayed physical development was referred: he was able to sit at 8 months of age, to stand at 12 months, to walk independently at 16 months and to have sphincter control at the age of 3 years. At 3 years, a neuropsychological evaluation revealed significant developmental delay with an overall functional level according to the Stanford Binet Intelligence Scale of a 2 year old child. He was referred as an “aggressive child” with semantic paraphasia.

At 2 years of age, an episode of loss of consciousness lasting 20 min was reported. Immediately after this episode, repeated bouts of hemiplegia of varying intensity involving either side of the body were reported. During most of the attacks, the patient presented with flaccid hemiplegia shifting sides, sometimes associated with tremor mostly involving upper limbs. Each of these attacks was associated with paroxysmal phenomena including dystonic posturing, which often appeared intermittently during the hemiplegic events. Choreaathetoid movements, nystagmus, and eye deviation were also seen during and between hemiplegic attacks, but no autonomic disturbances or history of seizure were reported. He never complained about headache during the attacks. All attacks were relieved by sleep.

Because of the first episode at 2 years of age, he was admitted to hospital and a computed tomography (CT) scan was performed, which was normal, and an electroencephalography (EEG) recording carried out during wakefulness and sleep. The EEG recording showed only mild asymmetry of the background activity with a focal slowing over central and centrotemporal regions predominating on the left side. The frequency of the subsequent attacks was approximately once to twice monthly, mostly evoked by emotional stress or by mild head trauma, the last happening a few months before the last neurological exam which was performed at the age of 12 years. At that time, mild increased tone was found, tendon reflexes were 3+ throughout, and a right extensor plantar response and a bilateral achilles clonus were present.

He is now clearly mentally retarded with lack of understandings of simple words or simple commands and an overall phenotype indicating severe neurological impairment.

**Subject III-3**

The subject is the third son, a 10 year old boy, born after a normal pregnancy and delivery. He presented delayed physical and mental development: he was able to sit at 15 months of age and started to walk independently at 18–20 months of age. Sphincter control was obtained at 3 years of age. At the age of 20 months he presented the first episode of flaccid areflexic paralysis involving both sides of the body, which lasted for a few hours, with no preceding seizures. In the following years, he presented a few rare episodes of generalised hypotonia and limb weakness while fully awake. Usually the attacks started with neck hypotonia followed by trunk muscle weakness and limb hypotonia. The sequence lasted several minutes and, during these episodes, the child had difficulty swallowing and eating and showed facial anemia. During and between these episodes autonomic disturbances were noticed similar to those seen in his oldest brother, but never nystagmus or other ocular abnormalities. No neuroradiological examination was performed. At the last neurological examination (at 7 years of age), there was evidence of mild mental retardation with learning disabilities and mild dysarthria but no other neurological signs.

**DNA extraction**

Following informed consent and approval by the ethical committee of the Scientific Institute E. Medea, blood samples were taken from all affected and non-affected members of the family and from 10 Italian sporadic AHC patients belonging to the Italian Association of AHC Syndrome (AISEA). Total DNA was extracted from 5 ml of blood with the IsoQuick Nucleic Acid Extraction kit (ORCA Research, Bothell, WA). A total of 125 healthy individuals (50 from the Greek population and 75 from the Italian population) were recruited as control subjects.

**Karyotype and haplotype analysis**

Karyotype analysis performed by standard procedure on all affected members of the family gave normal results.

Fluorescence-labelled oligonucleotides for polymorphic markers from the FHM2 locus and from 3p and 9q regions were designed based on the sequences available at the Human Genome Browser. Markers from 3p and 9q genomic regions were chosen to be approximately 1.5 Mb apart. PCR reactions were performed according to the melting temperature of each primer set used. PCR products were detected using an Applied Biosystems ABI 310 PE fluorescent sequencer and analysed with Genescan Analysis.
software (version 2.1, Applied Biosystems, Foster City, CA, USA).

Mutation analysis
Nucleotide numbers refer to the published cDNA sequence (NM_000702). Primers were designed to amplify all 23 exons covering the entire coding region, the exon–intron junctions, and variable portions of introns flanking each exon. Sequences were prepared with a BigDye Terminator Sequencing Kit (version 3.1, Applied Biosystems) run on an Applied Biosystems ABI 3100 Avant Genetic Analyzer and analysed with Sequencing Analysis Software, version 3.7 NT (Applied Biosystems, Foster City, CA, USA).

The mutation carrying fragment was amplified by the following set of primers: forward 5′-GGTCTAGGGTAAAGGTTATGG-3′; reverse 5′-CCCTCTTAATCTATTACCG-3′.

Constructs and site-directed mutagenesis
The full-length cDNA coding for the α2 wild-type and ouabain resistant constructs with and without the myc tag were described previously.11 We used the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) to

Figure 2  Sequence of DNA fragments containing the mutation identified and local amino acid sequence alignment of ATPases. (A) Electropherograms of genomic DNA fragment containing the mutation identified. Arrows show heterozygous 1237C→A transversion detected in AHC patients and the corresponding wild type sequence in control DNA. (B) Local amino acid sequence alignment of ATPases. Shown is the complete conservation of the T378 residue (arrow) in several subunits of different Na,K ATPases and H,K ATPases. The accession numbers are indicated on the left, while the amino acid position of the last residue in the alignment is shown on the right for each sequence.
introduce the C1237A mutation in both the myc-tagged and not-tagged ouabain resistant cDNAs.

All constructs were sequence verified.

**In vitro transcription and translation**

In vitro transcription and translation was performed using the TNT Coupled Reticulocyte Lysate System (Promega, Madison, WI, USA) and neosynthesised proteins were separated by SDS-PAGE (8%).

**Electrophoresis and Western blot analysis**

Proteins were resuspended in SDS-PAGE buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol) and separated for 2 h at 100 V in 8% SDS-polyacrylamide gels. Transblotted nitrocellulose membranes were incubated with monoclonal primary antibodies anti c-myc 9E10 (10 μg/ml) followed by incubation with horse-radish peroxidase (HRP)-conjugated secondary antibodies. Protein bands were visualised with the Enhanced Chemiluminescence Kit (Amersham Biosciences, Piscataway, NJ, USA).

**Transfections and ouabain treatment**

Constructs were transfected in HeLa cells by calcium phosphate following standard procedures. Cell viability was measured with the MTT reduction assay after 24 and 72 h of 1 μM ouabain challenge. We performed two independent experiments and in each experiment datapoints were in triplicate.

**Immunocytochemistry**

After transfection (48 h), COS-7 cells were fixed in 4% paraformaldehyde and incubated with monoclonal primary antibodies anti c-myc 9E10. Cells were then washed with PBS and incubated with Alexa Fluor 594-conjugated anti-mouse secondary antibody (Molecular Probes, Eugene, OR, USA). Cells were mounted in fluorescent mounting medium (DAKO, Glostrup, Denmark) and visualised under epifluorescence optics.

**Subcellular fractionation**

COS-7 cells were lysated on ice in 0.5 M NaCl, 10 mM Na2CO3, 0.1 mM PMSF, 10 mM COS-7 cells were lysated on ice in 0.5 M NaCl, 10 mM Subcellular fractionation recsence optics.

Cells were mounted in fluorescent mounting medium and incubated with Alexa Fluor 594-conjugated anti-mouse secondary antibodies.

**RESULTS**

**Haplotype analysis and mutation screening**

In order to verify the possible involvement of the genomic regions previously hypothesised by Mikati et al., namely 3p26 and 9q34, in generating the disease, we genotyped all family members with markers spanning a region of 9.5 Mb in about half of the introns of the gene (11 out of 23), the result was negative. As the set of primers used could detect heterozygosity at single nucleotide polymorphic sites present in the ATPases phosphorylation site (DKTGTIT, amino acids 374–380) of the hydro-

In the α subunit topology, the mutated residue resides in the large intracellular loop within the M4–M5 transmembrane segments (M1–M10). The affected residue is highly conserved in all known ε subunits of the Na,K and H,K pumps from vertebrates to invertebrates (fig 2B) suggesting a functional role in pump activity.

A search for mutations in the same gene was also conducted in a panel of 10 sporadic AHC patients, but the result was negative. As the set of primers used could detect heterozygosity at single nucleotide polymorphic sites present in the extracellular loop to confer resistance to ouabain, and the AHC mutation T378N. HeLa cells transfected with this construct did not survive to 1 μM ouabain challenge. The simulated heterozygous state, obtained by transfecting equal amounts of wild type and mutant cDNAs, showed intermediate behaviour (fig 3A). In addition, the mutant isoform showed rapid mortality typical of cells lacking Na,K ATPase pump activity (fig 3B).

To verify the expression constructs, we tested the wild type and mutant constructs by in vitro transcription and translation experiments. All the constructs gave the expected 112 kDa protein band, therefore excluding that a cloning artefact is responsible for the vulnerability of HeLa cells to ouabain when transfected with the mutant ATP1A2 cDNAs.

To confirm that the mutant AHC isoform is correctly located in the plasma membrane, COS-7 cells were transfected with wild type and the mutated constructs carrying an N-terminus myc tag. Immunofluorescence experiments show the same pattern of plasma membrane and cytoplasmic localisation for the wild type and the mutant isoform, thus excluding a possible retention of the mutant in the endoplasmic reticulum or other compartments. In addition, Western blot performed on microsomal and cytosolic fractions indicated that both the wild type and the AHC mutant constructs are detectable in the membrane fraction (not shown).

**DISCUSSION**

The clinical features of AHC and criteria for diagnosis include: onset before 18 months of age; repeated bouts of

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hemiplegia of varying intensity involving either or both sides of the body at least in some attacks; hemiplegic attacks precipitated by various factors including emotional triggers, head trauma, and fatigue; paroxysmal phenomena including tonic spells, dystonic posturing, choreoathetoid movements, nystagmus or other ocular abnormalities, and autonomic disturbances during and between hemiplegic attacks; attacks terminated by sleep; evidence of progression of mental and neurological deficits (reviewed in Rho and Chugani and the frequency of hemiplegic attacks, being in the lowest range (lower/equal than one/month), all together differentiate this family from most sporadic cases previously reported.4

In addition, we can detect a direct association between the severity of hemiplegic attacks and the grade of developmental delay and cognitive impairment, which represent two of the major manifestations of AHC syndrome. Indeed, although a progressive cognitive decline, associated with the disease, is
shared by all affected members of the family, it is clearly more severe in individuals III-1 and III-3 who had severe hemiplegic attacks, than in individuals II-4 and III-4.

In conclusion, the clinical findings observed in our AHC family confirm what has been previously described in the first family reported, a clearly milder phenotype shared by familial cases compared to sporadic cases. However, despite the clinical variability, all affected members of our family share the same novel mutation in the ATP1A2 gene.

The Na,K ATPase is a heteroeimeric enzyme comprised of a catalytic 112 KD a subunit, and b and g subunits. Four a subunit isoforms have been identified, each with unique tissue distribution: x1 is ubiquitous, x4 is restricted to testis, x3 is well represented in brain, and x2 is abundant in heart and brain, in both neurons and astrocytes. The AHC mutation described here is located in the largest intracellular loop of the x2 isoform between M4 and M5; this loop represents an important regulatory region for the ion transport system, harbouring the ATP-binding and hydrolysic domains. More precisely, the AHC mutation involves a highly conserved sequence, DKTGTL, which is the target of the b-aspartyl-phosphorylation reaction during catalysis in all P-type E1-E2-ATPases independently of their cation specificities.

According to this, the AHC mutation appears to cause a loss of function effect when transfected in HeLa cells, despite its correct localisation in the plasma membrane. Interestingly, a mutation in the same highly conserved esapeake sequence was found in the sarco-endoplasmic reticulum Ca2+ ATPase type 2 gene (ATP2A2) leading to Darier disease. The authors suggest that mutations in the ATP2A2 gene produce a dominant phenotype through haploinsufficiency.

Mutations in the ATP1A2 gene, but not in the same phosphorylation consensus sequence, have previously been recognised as responsible for familial hemiplegic migraine type II (FHM2), a disease with symptoms partially overlapping those of AHC, and FHM2 associated with benign familial infantile convulsions (BFIC). Also the FHM2 mutations described so far seem to have a loss of function effect when tested in eucaryotic cells, indicating that haploinsufficiency of ATP1A2 caused by a loss of a single allele leads to FHM2. The pathogenic mechanism leading to aura and migraine in FHM2 might involve two synergistic events: an increase in extracellular K+, producing wide cortical depolarisation, and an increase in intracellular Na+, which enhances intracellular Ca2+ through a decreased Na–Ca exchanger function. Supporting this is evidence that the x2 subunit distribution on the plasma membrane coincides with the localisation of the Na–Ca exchanger in neurons and astrocytes. At this point, the hypothesis formulated by Lance of AHC being a variant of migraine, could be better reformulated based on our findings, which demonstrate allelism between AHC and FHM2.

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ELECTRONIC-DATABASE INFORMATION

Electronic database information is as follows: Human Genome Browser: http://genome.ucsc.edu/; ATP1A2 cDNA: http://www.ncbi.nlm.nih.gov/
Here has been controversy about the usefulness of stratification by environmentally determined subgroups in genetic research. In cancer epidemiology only a few genetic polymorphisms cause a substantial change in risk and many genes involved in the metabolism of mutagens have little or no effect. But the inclusion of environmental measures in genetic studies might reveal environment-gene interactions. Such combined studies have been questioned, however, on the grounds that they are unlikely to increase the power of the genetic study very much. It has been said that the effects of genes in different environmental subgroups are likely to be in the same direction even if varying in size, and this would limit the gain in power. Australian authors have listed several examples to counter this argument.

The first example they give is of the influence of maternal smoking on the effect of the CYP1A1 gene on birthweight. The gene controls the metabolism of chemicals in tobacco smoke. When all 741 mothers in a study were considered the incidence of low birthweight was identical (21.5%) in mothers with genotype AA and mothers with genotype Aa or aa. Among the 124 maternal smokers however, the incidence of low birthweight was 24% (AA) v 45% (Aa or aa).

The second example concerns the GSTP1 glutathione transferase gene, Parkinson’s disease, and exposure to pesticides. Among all subjects in one study there was no significant association between the genotype and Parkinson’s disease but among subjects exposed to pesticides one polymorphism increased the risk of Parkinson’s disease more than fivefold. In the third example they quote a study of the effect of a monoamine oxidase A polymorphism on antisocial behaviour. It has been found that the polymorphism increases the rate of antisocial behaviour only among people who suffered from maltreatment as children. Among people who had not been maltreated in childhood the polymorphism tended to be associated with less antisocial behaviour. Another example of a change in direction of gene effect according to environmental subgroup is the effect of an alcohol dehydrogenase polymorphism on HDL cholesterol concentrations according to level of alcohol consumption. Different polymorphisms may also have different effects on the outcome of a single environmental factor. An example of this is the effect of different β2 adrenoceptor gene polymorphisms on the risk of obesity at varying levels of carbohydrate consumption.

The authors of this paper conclude that inclusion of environmental data may enhance the search for disease causing genes.

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