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De novo mutations in *ATP1A3* cause alternating hemiplegia of childhood

Alternating hemiplegia of childhood (AHC) is a rare, severe neurodevelopmental syndrome characterized by recurrent hemiplegic episodes and distinct neurological manifestations. AHC is usually a sporadic disorder and has unknown etiology. We used exome sequencing of seven patients with AHC and their unaffected parents to identify *de novo* nonsynonymous mutations in *ATP1A3* in all seven individuals. In a subsequent sequence analysis of *ATP1A3* in 98 other patients with AHC, we found that *ATP1A3* mutations were likely to be responsible for at least 74% of the cases; we also identified one inherited mutation in a case of familial AHC. Notably, most AHC cases are caused by one of seven recurrent *ATP1A3* mutations, one of which was observed in 36 patients. Unlike *ATP1A3* mutations that cause rapid-onset dystonia-parkinsonism, AHC-causing mutations in this gene caused consistent reductions in ATPase activity without affecting the level of protein expression. This work identifies *de novo ATP1A3* mutations as the primary cause of AHC and offers insight into disease pathophysiology by expanding the spectrum of phenotypes associated with mutations in *ATP1A3*.

AHC was first characterized as a distinct syndrome in 1971, with a report that described eight patients with episodes of intermittent hemiplegia on alternating sides of the body, developmental delay, dystonia and choreoathetosis beginning in infancy[1]. Since then, specific diagnostic criteria have more clearly defined the classic paroxysmal and interictal neurological manifestations associated with this disease[2–6]. AHC affects approximately 1 in 1 million individuals[7], with most cases occurring sporadically[5,8–10]. Although the etiology of AHC is usually unknown, a missense mutation in *ATP1A2* was reported in one case of atypical familial alternating hemiplegia[9,10]; however, the clinical presentation of some of the family members with the *ATP1A2* mutation was more consistent with familial hemiplegic migraine[9], which is caused by mutations in *ATP1A2* (refs. 11,12). To our knowledge, no cases of sporadic AHC have yet been attributed to *ATP1A2* mutations.

In this study, we used next-generation sequencing (NGS) of the exomes or whole genomes of ten individuals with AHC and, where possible, their unaffected parents. We identified and confirmed rare (minor allele frequency (MAF) <0.01%) mutations in *ATP1A3* (encoding the sodium-potassium (Na^+/K^+) ATPase α3 subunit, also known as ATP1A3) in eight of ten probands, and we showed that the mutations had occurred *de novo* in the seven patients for whom parental DNA was available. The *ATP1A3* mutations included five distinct nonsynonymous mutations, one of which was found in four patients with AHC (**Supplementary Table 1**). To further investigate *ATP1A3* in the two unexplained AHC probands, we looked for structural variants in the whole-genome sequence data, and we Sanger sequenced the protein-encoding exons to find single-nucleotide and insertion-deletion variants missed by whole-genome sequencing; neither analysis identified candidate causal *ATP1A3* mutations in these individuals. Given the rarity of functional *de novo* mutations, the occurrence of seven *de novo* mutations in the same gene in seven patients with AHC provides strong genetic evidence that mutations in *ATP1A3* cause sporadic AHC.

We next Sanger sequenced the protein-encoding exons of *ATP1A3* in an additional cohort of 95 individuals with AHC. In these 95 patients, we identified rare *ATP1A3* mutations in 74 patients (**Table 1**); these mutations were found to have arisen *de novo* in the 59 patients with sporadic AHC for whom parental DNA was available. Including samples sequenced with NGS, we identified a total of 19 different *ATP1A3* mutations in 82 of 105 (78%) patients studied. The majority of these mutations were located in or near regions encoding transmembrane domains (**Fig. 1**). Seven of the mutations were identified in multiple cases of AHC; in particular, those giving rise to amino acid substitutions D801N and E815K were identified in 36 (34%) patients and 19 (18%) patients, respectively (**Table 1**). One of the 95 patients evaluated had a familial form of alternating hemiplegia, first described in 1992 (ref. 8). In this individual, we identified a rare *ATP1A3* mutation (giving rise to I274N) affecting the encoded cytoplasmic domain that cosegregates with the AHC phenotype (**Fig. 2**; see **Supplementary Note** for phenotypic details of affected family members).

Thirteen of the 18 *ATP1A3* mutations seen in sporadic AHC cases were confirmed to be *de novo* (**Supplementary Table 1**). We also observed rare *ATP1A3* variants in 15 patients with sporadic AHC for whom parental DNA was not available, and it is possible that some of these mutations are inherited benign polymorphisms. This is unlikely given the rarity of functional variants in *ATP1A3*, but we can conservatively estimate the number of patients with pathogenic *ATP1A3* mutations by considering as pathogenic only those mutations observed as *de novo* in at least one patient. Under this criterion, 11 of the 15 patients have a pathogenic *ATP1A3* mutation. We can, therefore, conclude that at least 74% of patients with sporadic, typically presenting AHC studied here harbor disease-causing mutations in *ATP1A3*.

A full list of authors and affiliations appears at the end of the paper.

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Table 1 ***ATP1A3*** **mutations and protein modifications identified in AHC**

ATP1A3 modification[a]	Nucleotide change[a]	Number of AHC probands with the mutation
S137Y	c.410C>A	2
S137F	c.410C>T	1
Q140L	c.419A>T	1
D220N	c.658G>A	1
I274N	c.821T>A	1
C333F	c.998G>T	2
G755S	c.2263G>A	1
N773S	c.2318A>G	1
D801N	c.2401G>A	36
M806R	c.2417T>G	1
I810S	c.2429T>G	1
S811P	c.2431T>C	4
E815K	c.2443G>A	19
Splice site	c.2542–1:G>A	1
V919del	c.2755_2757delGTC	1
G947R	c.2839G>A	5
G947R	c.2839G>C	2
A955D	c.2864C>A	1
D992Y	c.2974G>T	1

[a]*ATP1A3* mutations and encoded protein coordinates are defined on the basis of UniProt ID P13637 (ref. 24) and Consensus CDS ID CCDS12594.1 (ref. 25).

ATP1A3 is an α subunit of the Na^+/K^+ ATPase pump that is partly responsible for establishing and maintaining electrochemical gradients of sodium and potassium ions across the plasma membrane of neurons[13]. Mutations in *ATP1A3* have been shown to cause rapid-onset dystonia-parkinsonism (DYT12)[14–18]. None of the mutations known to cause DYT12 was found in patients with AHC; however, two AHC-causing mutations (encoding D801N and I274N) affect amino acids also affected by DYT12-causing mutations.

To better understand how *ATP1A3* mutations cause two clinically distinct disorders, we studied the *in vitro* functional consequences of the five mutations identified in the NGS screens of patients with AHC and ten mutations that cause DYT12. All 15 mutations were introduced in expression constructs, transfected into COS-7 and HeLa cells, and assessed for *ATP1A3* expression and function. None of the mutations was found to affect *ATP1A3* mRNA expression (**Supplementary Fig. 1**).

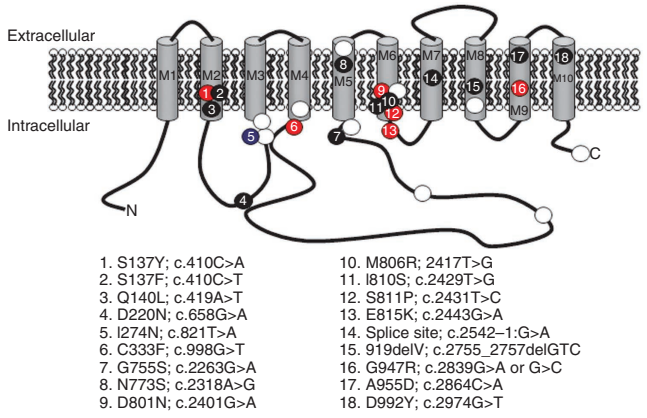

Figure 1 *ATP1A3* mutations and encoded protein modifications associated with AHC and DYT12. Diagram of the ATP1A3 protein shows positions of protein modifications associated with DYT12 (white dots)[23], or with a single case (black dots), multiple cases (red dots) and a familial case (blue dot) of AHC. Lower panel, *ATP1A3* mutation coordinates defined on the basis of UniProt ID P13637 (ref. 24) and Consensus CDS ID CCDS12594.1 (ref. 25).

However, seven out of ten mutations that cause DYT12 reduced ATP1A3 protein expression to undetectable levels, whereas none of the AHC-causing mutations reduced protein expression compared to wild-type *ATP1A3* (**Fig. 3** and **Supplementary Fig. 2**). Despite having different effects on protein expression, both mutations that cause AHC and those that cause DYT12 reduced ATP1A3 *in vitro* by 54–90% (**Fig. 3**). We note that the previous study of ATP1A3 expression in DYT12 reported that protein expression was attenuated by the D801Y substitution and not affected by the I274T substitution[14], whereas we observe opposite effects in our study. This discrepancy may be attributed to different sensitivities of the assay in specific cell lines. Despite this inconsistency, DYT12-causing mutations typically reduced ATP1A3 protein expression in both studies, whereas in our study no AHC-causing mutation reduced protein levels. Because hypomorphic mutations would lead to reduced protein abundance and modifications that are distributed throughout the protein rather than concentrated in transmembrane domains, these data suggest that *ATP1A3* mutations causing AHC modulate the Na^+/K^+ ATPase pump's activity, whereas DYT12-causing mutations do so through hypomorphic effects on the pump. Further supporting this hypothesis, evaluation of the crystal structure of the Na^+/K^+ ATPase[19] predicts that a D801N substitution in AHC will prevent the binding of potassium ions to the Na^+/K^+ ATPase pump. One possible exception to this pattern is the *de novo* splice-site mutation observed in a patient with AHC, which may result in protein elimination by a frameshift, although it could also result in a protein with altered activity.

As noted above, seven of the 19 AHC-causing mutations were observed in multiple patients. A recurrent mutation in *FGFR3* was previously reported to cause the majority of cases of achondroplasia[20], suggesting the presence of a hypermutable sequence in that gene. In AHC, the recurrence of *de novo* mutations may be a result of hypermutable sequences in *ATP1A3*, the ascertainment effect of only a specific subset of mutations causing AHC, or both. Some contribution of hypermutability is indicated by the following analysis: we observed 13 sites that carry *de novo* mutations in *ATP1A3*; under the null hypothesis that the mutation rate is equal among these sites, the chance that any single mutation would be seen in at least 36 of the total 77 patients found to have a 'pathogenic' mutation (as observed for the mutation giving rise to D801N) is low ($P < 0.0001$). Furthermore, three of the seven recurrently mutated sites found in AHC are G>A substitutions

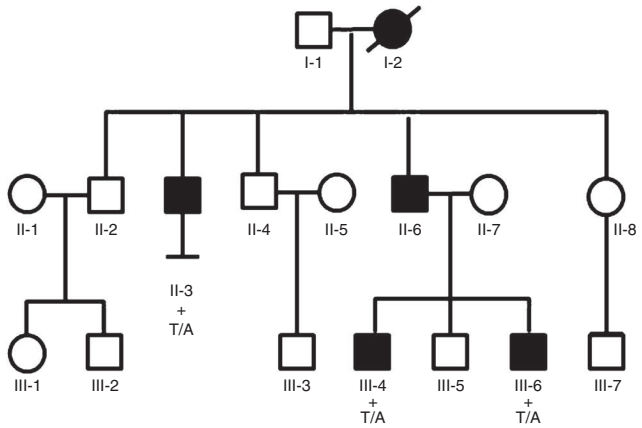

Figure 2 Pedigree of a family with autosomal dominant AHC[1]. Affected individuals indicated by black shading. An *ATP1A3* mutation (c.821T>A; I274N) was identified in patients II-3, III-4 and III-6 (+). DNA was unavailable from subjects II-6 and I-2. See **Supplementary Note** for phenotypic details of affected patients.

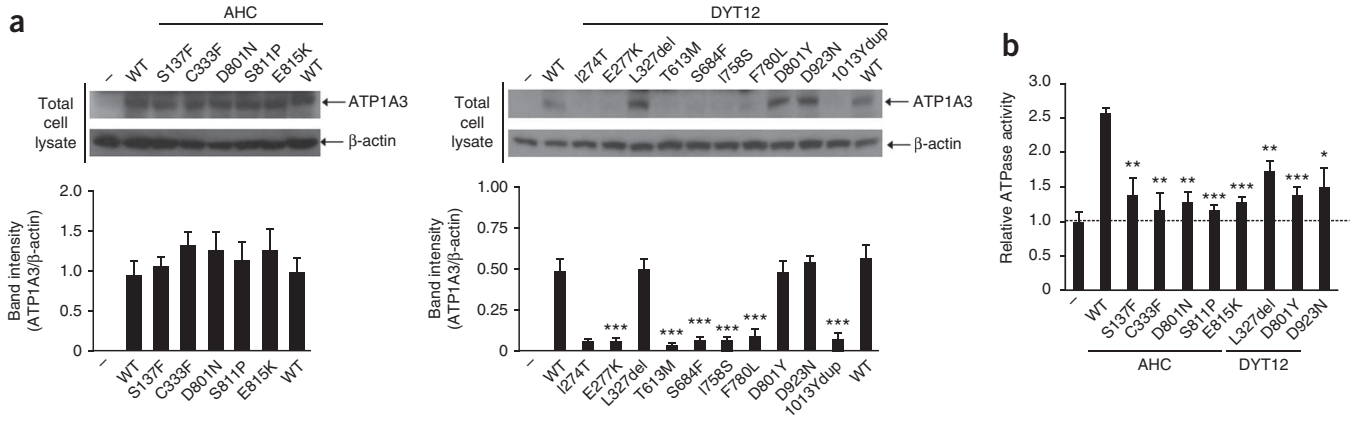

Figure 3 Effects of disease-causing mutations on protein expression and enzyme activity in COS-7 cells. (**a**) Protein-blot analysis of ATP1A3 abundance in untransfected cells (–) or cells expressing wild-type (WT) *ATP1A3* or AHC- or DYT12-causing *ATP1A3* mutations. Lower panels show relative protein expression as band intensity compared to β-actin. (**b**) ATPase activity in cells transfected with mutant or WT *ATP1A3* relative to untransfected cells (–). Error bars, mean ± s.d.; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, Student's *t*-test (uncorrected for multiple testing).

occurring at hypermutable methylated CpG-dinucleotide sequences[21] (**Table 1**). It also seems that only a specific subset of *ATP1A3* mutations lead to AHC, as nearly all identified AHC-causing mutations affect regions in or near transmembrane domains of the encoded protein, whereas the DYT12 mutations are more evenly distributed (**Fig. 1**). These results suggest that the observed patterns of AHC-causing mutations across *ATP1A3* result from hypermutable sequences and that only a small set of specific mutations can cause AHC. This is consistent with functional analyses suggesting that AHC-causing mutations may have specific effects on protein function rather than simply decreasing protein activity. Although not done in this study, functional evaluation of the inherited *ATP1A3* mutation (giving rise to I124N) may reveal distinct effects on the activity of Na^+/K^+ ATPase that may help to explain the atypical familial AHC phenotype.

Myshkin mice, which are heterozygous for a missense *Atp1a3* mutation (encoding a I810N substitution) that inactivates Na^+/K^+ ATPase, are predisposed to seizure activity that can be mitigated by replacement of the gene with functional *Atp1a3* (ref. 22). We therefore evaluated whether patients with *ATP1A3* mutations were more likely to have seizures than those without, and found a minor but significant effect (54% of those with *ATP1A3* mutations had a history of seizures, compared to 29% of those without; $P = 0.01$, binomial probability calculation). For one patient cohort ($n = 30$) for which consistent phenotyping had been done[5], we also compared age at first paroxystic event, age at first hemiplegic attack and a series of disability indices but found no statistically significant differences between those with and without *ATP1A3* mutations. More detailed investigations are needed to characterize the phenotypic spectrum associated with *ATP1A3* mutations and to compare phenotypes among patients with different *ATP1A3* mutations.

Mutations in *ATP1A3* probably account for at least 74% of cases of typical sporadic AHC. Because the present study assessed only patients with typical AHC, further work will be needed to assess whether *ATP1A3* mutations cause distinct, but related, conditions. In addition to identifying the cause of the majority of AHC cases, our results now implicate another clinically distinct disease linked to *ATP1A3* and open the door to detailed functional characterization of mutations that cause the different diseases. The ability to test the functional consequences of two groups of mutations that lead to clinically distinct phenotypes will offer insight into the pathophysiological processes unique to each disease and could facilitate drug discovery for these and related conditions.

URLs. Ensembl database, http://www.ensembl.org; DYT12 modifications compiled from Human Gene Mutation Database (HGMD), http://www.hgmd.org; I.B.AHC Biobank and Clinical Registry for Alternating Hemiplegia, http://en.ibahc.org; ImageJ software, http://rsbweb.nih.gov/ij/; National Heart, Lung, and Blood Institute (NHLBI) Exome Sequencing Project (ESP) Exome Variant Server, http://evs.gs.washington.edu/EVS; SequenceVariantAnalyzer (SVA), http://www.svaproject.org; Consensus CDS database, http://www.ncbi.nlm.nih.gov/CCDS; UniProt Knowledgebase (UniProtKB), http://www.uniprot.org/uniprot/.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

E.L.H., Y.H., S.M.S., M.A.M. and D.B.G. conceived and designed the study. Genetic data were generated and analyzed by E.L.H., K.J.S., Y.H., F.G., S.N., B.d.V., F.D.T., S.F., E.A., L.D.P., C.H., L.B.J., K.V.S., C.E.G., L.L., G.N., A.A. A.M.J.M.v.d.M and D.B.G. DNA samples and phenotypic information for AHC patients were collected, compiled and analyzed by K.J.S., F.G., S.N., N.M.W., B.d.V., F.D.T., B.F., S.H., E.P., M.T.S., T.M.N., L.V., S.P.R., K.J.M., K.S., L.J.P., J.H., M.D.F., A.M.B., G.K.H., C.M.W., D.W., B.J.L., P.U., M.D.K., I.E.S., G.N., A.A., S.M.S., M.A.M., the European AHC Genetics Consortium, the I.B.AHC Consortium and the ENRAH for SMEs Consortium. E.L.H., A.M.J.M.v.d.M., S.M.S., M.A.M. and D.B.G. wrote the paper. All authors reviewed the compiled manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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Erin L Heinzen[1,2,35], Kathryn J Swoboda[3,4,35], Yuki Hitomi[1,35], Fiorella Gurrieri[5], Sophie Nicole[6–8], Boukje de Vries[9], F Danilo Tiziano[5], Bertrand Fontaine[6–8,10], Nicole M Walley[1], Sinéad Heavin[11], Eleni Panagiotakaki[12], the European Alternating Hemiplegia of Childhood (AHC) Genetics Consortium[13], the Biobanca e Registro Clinico per l'Emiplegia Alternante (I.B.AHC) Consortium[13], the European Network for Research on Alternating Hemiplegia (ENRAH) for Small and Medium-sized Enterpriese (SMEs) Consortium[13], Stefania Fiori[5], Emanuela Abiusi[5], Lorena Di Pietro[5], Matthew T Sweney[3], Tara M Newcomb[3], Louis Viollet[4], Chad Huff[14], Lynn B Jorde[14], Sandra P Reyna[4], Kelley J Murphy[4], Kevin V Shianna[1,2], Curtis E Gumbs[1], Latasha Little[1], Kenneth Silver[15,16], Louis J Ptáček[17,18], Joost Haan[19,20], Michel D Ferrari[20], Ann M Bye[21], Geoffrey K Herkes[22], Charlotte M Whitelaw[23], David Webb[24], Bryan J Lynch[25], Peter Uldall[26], Mary D King[25], Ingrid E Scheffer[11,27,28], Giovanni Neri[5], Alexis Arzimanoglou[12,29,30], Arn M J M van den Maagdenberg[9,20], Sanjay M Sisodiya[31,36], Mohamad A Mikati[32,33,36] & David B Goldstein[1,34,36]

[1]Center for Human Genome Variation, Duke University School of Medicine, Durham, North Carolina, USA. [2]Department of Medicine, Duke University School of Medicine, Durham, North Carolina, USA. [3]Department of Pediatrics, University of Utah, Salt Lake City, Utah, USA. [4]Department of Neurology, University of Utah, Salt Lake City, Utah, USA. [5]Instituto di Genetica Medica, Università Cattolica del Sacro Cuore, Policlinico A. Gemelli, Rome, Italy. [6]Université Pierre et Marie Curie, Centre de Recherche de l'Institut du Cerveau et de la Moelle épinière UMR S975, Paris, France. [7]Institut National de la Santé et de la Recherche Médicale, U975, Paris, France. [8]Centre National de la Recherche Scientifique, UMR7225, Paris, France. [9]Department of Human Genetics, Leiden University Medical Centre, Leiden, The Netherlands. [10]Assistance Publique-Hôpitaux de Paris, Département de Neurologie & Centre de Référence Canalopathies Musculaires, Groupe Hospitalier de la Pitié-Salpêtrière, Paris, France. [11]Department of Medicine, University of Melbourne, Austin Health, Melbourne, Australia. [12]Epilepsy, Sleep and Pediatric Neurophysiology Department, Woman-Mother-Child Hospital, University Hospitals of Lyon (HCL), Lyon, France. [13]A full list of members and affiliations appears at the end of this paper. [14]Department of Human Genetics, Eccles Institute of Human Genetics, University of Utah, Salt Lake City, Utah, USA. [15]Department of Neurology, Comer Children's Hospital, University of Chicago, Chicago, Illinois, USA. [16]Department of Pediatrics, Comer Children's Hospital, University of Chicago, Chicago,

Illinois, USA. [17]Department of Neurology, University of California, San Francisco, San Francisco, California, USA. [18]Howard Hughes Medical Institute, University of California, San Francisco, San Francisco, California, USA. [19]Department of Neurology, Rijnland Hospital, Leiderdorp, The Netherlands. [20]Department of Neurology, Leiden University Medical Center, Leiden, The Netherlands. [21]Department of Paediatric Neurology, Sydney Children's Hospital, Randwick, New South Wales, Australia. [22]University of Sydney, Royal North Shore Hospital, St. Leonards, Sydney, New South Wales, Australia. [23]Northern Clinical School, University of Sydney, and Royal North Shore Hospital, Sydney, Australia. [24]Our Lady's Children's Hospital, Crumlin, Dublin, Ireland. [25]The Children's University Hospital, Temple Street, Dublin, Ireland. [26]Department of Paediatrics and Adolescent Medicine, Rigshospitalet, University of Copenhagen, Copenhagen, Denmark. [27]Department of Paediatrics, University of Melbourne, Royal Children's Hospital, Melbourne, Australia. [28]Florey Neuroscience Institutes, Melbourne, Australia. [29]Institut National de la Santé et de la Recherche Médicale, U1028, Centre de Recherche en Neurosciences de Lyon, Lyon, France. [30]Centre National de la Recherche Scientifique, UMR 5292, Lyon, France. [31]Department of Clinical and Experimental Epilepsy, University College London Institute of Neurology, London, UK. [32]Division of Pediatric Neurology, Duke University Medical Center, Durham, North Carolina, USA. [33]Department of Neurobiology, Duke University, Durham, North Carolina, USA. [34]Department of Molecular Genetics and Microbiology, Duke University School of Medicine, Durham, North Carolina, USA. [35]These authors contributed equally to this work. [36]These authors jointly directed this work.

European Alternating Hemiplegia of Childhood (AHC) Genetics Consortium

Sophie Nicole[6–8], Fiorella Gurrieri[5], Giovanni Neri[5], Boukje de Vries[9], Stephany Koelewijn[9], Jessica Kamphorst[9], Marije Geilenkirchen[9], Nadine Pelzer[20], Laura Laan[20], Joost Haan[19,20], Michel Ferrari[20] & Arn van den Maagdenberg[9,20]

Biobanca e Registro Clinico per l'Emiplegia Alternante (I.B.AHC) Consortium

Claudio Zucca[37], Maria Teresa Bassi[38], Filippo Franchini[39], Rosaria Vavassori[39], Melania Giannotta[40], Giuseppe Gobbi[40], Tiziana Granata[41], Nardo Nardocci[41], Elisa De Grandis[42], Edvige Veneselli[42], Michela Stagnaro[42], Fiorella Gurrieri[5], Giovanni Neri[5] & Federico Vigevano[43]

European Network for Research on Alternating Hemiplegia (ENRAH) for Small & Medium-sized Enterprises (SMEs) Consortium

Eleni Panagiotakaki[12], Claudia Oechsler[44], Alexis Arzimanoglou[12,29,30], Sophie Nicole[6–8], Melania Giannotta[40], Giuseppe Gobbi[40], Miriam Ninan[45], Brian Neville[45], Friedrich Ebinger[44], Carmen Fons[46], Jaume Campistol[46], David Kemlink[47], Sona Nevsimalova[47], Laura Laan[20], Cacha Peeters-Scholte[20], Arn van den Maagdenberg[9,20], Paul Casaer[48], Giorgio Casari[49,50], Guenter Sange[51], Georg Spiel[51], Filippo Martinelli Boneschi[49,50], Claudio Zucca[37], Maria Teresa Bassi[38], Tsveta Schyns[52], Francis Crawley[53], Dominique Poncelin[54] & Rosaria Vavassori[39]

[37]Clinical Neurophysiology Unit, Scientific Institute E. Medea, Lecco, Italy. [38]Laboratory of Molecular Biology, Scientific Institute E. Medea, Lecco, Italy. [39]Associazione Italiana per la Sindrome di Emiplegia Alternante (AISEA) Onlus, Lecco, Italy. [40]Child Neurology Unit, Maggiore Hospital, Bologna, Italy. [41]Department of Child Neurology, National Neurological Institute C. Besta, Milan, Italy. [42]Department of Child Neuropsychiatry, G. Gaslini Hospital, University of Genoa, Genoa, Italy. [43]Division of Neurology, Bambino Gesù Children's Hospital, Rome, Italy. [44]Department of Child Neurology, Heidelberg University Hospital, Heidelberg, Germany. [45]Neurosciences Unit, University College London Institute of Child Health, London, UK. [46]Department of Child Neurology, Sant Joan de Déu Hospital, Barcelona, Spain. [47]Department of Neurology, Charles University, First Faculty of Medicine and Teaching Hospital, Prague, Czech Republic. [48]Department of Child Neurology, University Hospital Gasthuisberg, Leuven, Belgium. [49]Department of Neurology, Division of Neuroscience, San Raffaele Scientific Institute, Milan, Italy. [50]Institute of Experimental Neurology (INSPE), San Raffaele Scientific Institute, Milan, Italy. [51]Department of Neurology and Psychiatry of Children and Adolescents, General Hospital, Klagenfurt, Austria. [52]The ENRAH Association, Brussels, Belgium. [53]Good Clinical Practice Alliance—Europe (GCPA), Kessel-Lo, Belgium. [54]Association Française de l'Hémiplégie Alternante (AFHA), St. Germain les Arpajon, France. Correspondence should be addressed to D.B.G. (d.goldstein@dm.duke.edu) or M.A.M. (mohamad.mikati@dm.duke.edu).

ONLINE METHODS

Study population. All sequenced patients met the diagnostic criteria for typical AHC[4]. Exome- and genome-sequenced patients were recruited to take part in this study through the Genetics of Epilepsy study at Duke University Medical Center and the Genetics of Epilepsy study at University College London and the Pediatric Motor Disorders Research Program at the University of Utah. Blood samples for DNA extraction were collected from each affected child and, when possible, from unaffected parents. Patients in the follow-up cohort were obtained from Duke University Medical Center ($n = 2$), the I.B.AHC Biobank and Clinical Registry for Alternating Hemiplegia ($n = 34$), the European AHC Genetics Consortium ($n = 16$), the University of Melbourne ($n = 7$), the University of Utah ($n = 3$ related patients with familial AHC; **Fig. 2**), the French DNA and Cell Biobank for AHC ($n = 30$), Our Lady's Children's Hospital (Dublin, Ireland; $n = 4$) and the Children's University Hospital (Dublin, Ireland; $n = 1$). All patients were recruited and consent was obtained according to the standards set forth by the ethics boards at the collection sites. Detailed phenotypic and demographic information of AHC patients with *ATP1A3* mutations are provided in **Supplementary Table 1**.

Next-generation sequencing. Samples were either exome sequenced using Agilent's All Exon (50 MB) capture or whole-genome sequenced. All exome and whole-genome sequencing was performed on either Illumina GAIIx or HiSeq 2000 machines in the Genomic Analysis Facility at the Center for Human Genome Variation (Duke University). Sequence data from 484 controls nonenriched for neuropsychiatric diseases that were sequenced as part of other in-house studies (for example, genetics of cognition, birth weight and HIV resistance) were used to ascertain candidate variant genotype frequencies. Sequencing was performed using standard protocols. The targeted exonic regions of all exome-sequenced AHC-affected individuals and their parents were sequenced to an average coverage of 90-fold (minimum 65-fold), with at least 95% of the captured region having >5-fold coverage. Whole-genome sequenced patient samples were sequenced to an average coverage of greater than 25-fold.

Paired-end reads were aligned to the Human Reference Genome (NCBI Build 36) using BWA software[26]. Variant calling to detect single-nucleotide variants and insertions or deletions was performed using SAMtools software[27]. Structural variation was called from whole-genome sequenced samples using software developed in the Center for Human Genome Variation, ERDS[28] and SV-Finder. SequenceVariantAnalyzer (SVA)[29] was used to annotate variants identified from the sequence data (Ensembl 50_36l). This software provides each variant with a genomic context (such as nonsynonymous coding versus splice site, gene name, transcript and associated GO term)[29]. Identity-by-descent calculations were used to confirm paternity and maternity.

Pictures of aligned NGS fragments covering the *de novo ATP1A3* mutations in the seven sequenced AHC trios (parents and affected child) are provided in **Supplementary Figure 3**.

Prioritization of candidate disease-causing mutations. Genotypes of variants identified in each of the seven patients were evaluated for presence in their unaffected parents and also in a set of 484 population controls. Any genotype identified in either the unaffected parent or a population control was assumed to be noncausal. Genotypes present in the patients and absent in the unaffected parents, 484 sequenced controls and 5,400 individuals sequenced as part of the NHLBI Exome Sequencing Project (ESP) Exome Variant Server (v.0.0.8, release ESP5400) were considered as possibly causal.

Candidate disease-causing mutations were confirmed to be both present and *de novo* using Sanger sequencing.

Sanger sequencing of the protein-encoding exons of *ATP1A3*. Some or all of the protein-encoding exons of *ATP1A3* were Sanger sequenced in a follow-up cohort of 95 AHC patients using standard methods. When parental DNA samples were not available, heterozygous mutations absent in any publicly available database were presumed to be causal (confirmed *de novo* status is noted in **Supplementary Table 1**). Sequencing primers are available upon request. We compared the sample sources between clinical sites to ensure no overlap. For 59 of the 70 AHC patients with recurrent *ATP1A3* mutations, we genotyped 10 common polymorphisms to confirm that samples harboring the same *ATP1A3* mutation were unique.

Functional characterization of disease-causing mutations. Pathogenic mutations in *ATP1A3* identified in this study and a series of mutations previously implicated in DYT12 (refs. 14–18) were evaluated for effects on protein expression and overall ATPase activity *in vitro*.

Plasmids. Human *APT1A3* cDNA samples were amplified in four fragments from first-strand cDNA derived from total RNA extracted from the human neuroblastoma-derived cell line A172. Each part of *ATP1A3* cDNA was then subcloned into the pCR-Blunt II-TOPO vector (Invitrogen–Life Technologies). Fifteen different mutant alleles were produced by PCR-directed mutagenesis and the sequences were confirmed. The wild-type or mutant cDNA parts were then subcloned into pCR-Blunt II to generate full-length cDNAs. Each full-length *ATP1A3* cDNA (wild-type and 15 mutants) was then subcloned into the expression vector pcDNA3.1(+). The sequences of the constructs were confirmed by sequence analysis. Primer sequences used for the generation of the plasmids are available upon request.

Quantitative RT-PCR. Empty pcDNA3.1(+) vector, pcDNA3.1(+)-*ATP1A3*–wild-type vector and pcDNA3.1(+)-*ATP1A3* vectors for each mutant allele were transfected into HeLa cells by lipofection using Lipofectamine 2000 (Invitrogen–Life Technologies). Total RNA was extracted from transfectant, and cDNAs were synthesized. *ATP1A3* and *GAPDH* mRNA expression was estimated using RT-PCR (primer sequences available upon request).

Protein blotting. Empty pcDNA3.1(+) vector, pcDNA3.1(+)-ATP1A3–wild-type vector, and each of the pcDNA3.1(+)-ATP1A3–mutant allele vectors were transfected into HeLa and COS-7 cells by lipofection. After 48 h of transfection, the cell lysates were separated on an SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane (Millipore). The membranes were then incubated with a monoclonal antibody to human-ATP1A3 (1:3,000; Santa Cruz Biotechnology, sc-374050) or an antibody to β-actin (1:5,000; Santa Cruz Biotechnology, sc-47778). Proteins were visualized using enhanced chemiluminescence with a protein-blotting detection (GE Healthcare). The effects of the mutations on protein abundance were quantified using ImageJ software.

ATPase assay. COS-7 cells expressing wild-type or mutant alleles of *ATP1A3* were lysed gently by Mammalian Protein Extraction Buffer (GE Healthcare) and protease inhibitor cocktail (Sigma-Aldrich). Only mutant alleles expressing detectable ATP1A3 protein levels in the protein-blot assay were assessed in the ATPase assay. COS-7 cells were chosen for the ATPase analysis because HeLa cells do not express *ATP1A2*, which encodes an essential subunit of the Na^+/K^+ ATPase. The lysates were then incubated with 250 μM of ATP, 40 mM of NaCl, 25 mM of KCl, 3 mM of $MgCl_2$ and 1 mM ethylene glycol tetra-acetic acid in 37 °C for 1 h. Synthesized ADP by ATPase reaction was detected by ADP-Glo Kinase Assay (Promega). ATPase activity was estimated by subtracting the luminescence signal with the addition of 100 μM of ouabain from the luminescence reading without ouabain.

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