Exome sequencing of ATP1A3-negative cases of alternating hemiplegia of childhood reveals SCN2A as a novel causative gene

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ARTICLE

Exome sequencing of *ATP1A3*-negative cases of alternating hemiplegia of childhood reveals *SCN2A* as a novel causative gene

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Alternating hemiplegia of childhood (AHC) is a rare neurodevelopment disorder that is typically characterized by debilitating episodic attacks of hemiplegia, seizures, and intellectual disability. Over 85% of individuals with AHC have a de novo missense variant in *ATP1A3* encoding the catalytic α3 subunit of neuronal Na^{+}/K^{+} ATPases. The remainder of the patients are genetically unexplained. Here, we used next-generation sequencing to search for the genetic cause of 26 *ATP1A3*-negative index patients with a clinical presentation of AHC or an AHC-like phenotype. Three patients had affected siblings. Using targeted sequencing of exonic, intronic, and flanking regions of *ATP1A3* in 22 of the 26 index patients, we found no ultra-rare variants. Using exome sequencing, we identified the likely genetic diagnosis in 9 probands (35%) in five genes, including *RHOBTB2* ($n = 3$), *ATP1A2* ($n = 3$), *ANK3* ($n = 1$), *SCN2A* ($n = 1$), and *CHD2* ($n = 1$). In follow-up investigations, two additional *ATP1A3*-negative individuals were found to have rare missense *SCN2A* variants, including one de novo likely pathogenic variant and one likely pathogenic variant for which inheritance could not be determined. Functional evaluation of the variants identified in *SCN2A* and *ATP1A2* supports the pathogenicity of the identified variants. Our data show that genetic variants in various neurodevelopmental genes, including *SCN2A*, lead to AHC or AHC-like presentation. Still, the majority of *ATP1A3*-negative AHC or AHC-like patients remain unexplained, suggesting that other mutational mechanisms may account for the phenotype or that cases may be explained by oligo- or polygenic risk factors.

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INTRODUCTION

Alternating hemiplegia of childhood (AHC) is a rare, early-onset neurodevelopmental disorder with a distinctive clinical presentation, first described in 1971, that involves episodic hemiplegia that can alternate between sides of the body [1]. These individuals may also have episodes of quadriplegia, paroxysmal attacks of dystonia, abnormal eye movements, and/or autonomic dysfunction, and movement disorders such as chorea, dystonia, and intellectual disability [1–5]. In 2012, de novo variants in *ATP1A3* were found to explain the vast majority (~85%) of cases of AHC, providing

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evidence of the early clinical hypotheses that suggested a distinct monogenic phenotype [6, 7]. Still, a number of patients who fulfill the clinical diagnostic criteria of AHC remain genetically unexplained. As comprehensive sequencing of the coding regions of *ATP1A3* had not identified a causal mutation, these cases are referred to as *ATP1A3*-negative, either typical AHC patients or patients with one or more of the hallmark clinical traits observed in the condition (AHC-like). Exome sequencing in parallel studies has shown that rare protein-disrupting variants in *ATP1A2* [8, 9] and *RHOBTB2* [10, 11] contribute to AHC, although these collectively explain a very small fraction of cases. Hence we here sought to (1) use targeted whole-gene sequencing of *ATP1A3* to look for non-coding pathogenic variants in our cohort of 26 *ATP1A3*-negative patients, and (2) use exome sequencing to further identify other genes that may be responsible for the observed phenotypes.

MATERIALS (SUBJECTS) AND METHODS

Study participants

Twenty-six individuals with typical AHC (or AHC-like) were consented and enrolled at either Duke University, University Hospitals of Lyon, Catholic University in Rome, or Leiden University Medical Center. Unaffected parents were also collected for 20 of the probands (Table 1). Seventeen probands were sporadic, one proband had two additional affected full siblings (quintet), one proband had one affected sibling (quad), and one proband had an affected half-sibling (Fig. 1). Probands were classified as "typical" if they fulfilled the diagnostic criteria [2]. In all cases, only one affected child underwent targeted gene or exome sequencing. DNA was extracted from blood or saliva from the proband and unaffected parents for genomic analyses at the referral Centers. A control cohort consisted of 11,151 individuals sequenced as part of other genetics studies in the Institute for Genomic Medicine (Columbia University, New York, NY, USA) was used to exclude site-specific artifacts and ascertain variant frequency in the population. This cohort consisted of healthy individuals or individuals with phenotypes unrelated to neurodevelopmental disorders.

Following the analysis of the exome sequence data, two additional *ATP1A3*-negative AHC cases (IT06 and 13A2344) were identified with rare missense *SCN2A* variants. These individuals were sequenced and analyzed as part of other studies. Since they were not analyzed as part of the initial cohort, these are being reported as secondary findings. Phenotypic information based on presence or absence of the six core features of AHC [2] is provided for all probands and affected family members ($n = 32$) (Supplementary Table 1).

Targeted and exome sequencing and data processing

DNA samples from all 26 probands and parents underwent exome sequencing using either the IDT xGen Exome Research Panel v1 (IDT Corporation, Newark, New Jersey, USA) or the Nimblegen SeqCap EZ V3.0 Exome Enrichment Kit (NimbleGen Systems GmbH, Pleiskirchen, Germany) per protocol. A subset of 22 probands also underwent targeted sequenced on a custom-designed panel (Nimblegen SeqCap EZ V3.0 Custom Enrichment Kit; NimbleGen Systems GmbH, Pleiskirchen, Germany) that included the full gene sequence of *ATP1A3*, including protein-coding, non-coding, and 1 kb up- and downstream of the protein-coding sequence. The other four samples from the exome-sequenced cohort did not have sufficient DNA available for such targeted deep sequencing of *ATP1A3*. Sequencing was performed on the HiSeq2500 and NovaSeq platforms (Illumina, Inc., San Diego, CA, USA). The sequenced fragments were aligned with DRAGEN to hg19/ GRCh37. Variants were called using the Genome Analysis Toolkit (GATK) v3.6 best practices [12]. Variants were annotated using consensus coding sequence (release 20) using snpEff [13]. Genomic analyses were performed using Analysis Tool for Annotated Variants [14].

Two additional cases with an *SCN2A* variant were identified separately from the 26 exome-sequenced probands. One case harboring the de novo variant in *SCN2A* underwent exome sequencing at Catholic University in Rome using the Ion AmpliSeq™ Exome RDY Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Sequencing was performed by the ION-Proton Instrument (Thermo Fisher Scientific, Waltham, MA, USA). Raw data were aligned to the hg19 by the Torrent Suite (v.5.0.4). Following the upload of the BAM file into the Ionreporter cloud tool, variants were identified by the Variant Caller Plugin (v. 5.10). The second individual, who was found to have an *SCN2A* variant, underwent targeted sequencing using a custom Nimblegen SeqCap EZ V3.0 Custom Enrichment Kit (NimbleGen Systems GmbH, Pleiskirchen, Germany) that included the protein-coding regions of *SCN2A*. Parental DNA was unavailable in this case to ascertain inheritance.

Calling of rare variants and genotypes from exome and target sequencing in probands

De novo variants were identified from the annotated variant lists using the following criteria: (1) heterozygous variant call in the proband with at least 10-fold coverage; (2) exclude variants with a minor allele frequency (MAF) > 0% in the Institute of Genomic Medicine control cohort, and in Exome Variant Server (EVS), Exome Aggregate Consortium (ExAC release 0.3), and gnomAD browser (v2.1.1); (3) exclude variants with a GATK RMSMapping-Quality score of <40, QualbyDepth Score <2, quality score <50; (4) exclude variants with a variant allele fraction of <30% and <70%; (5) exclude variants in RepeatMasker regions (RepeatMasker 4.1.0, http://www.repeatmasker.org); (6) in cases where sequencing of unaffected was performed, the parents were required to have a homozygous reference genotype with at least 10-fold coverage; and (7) for exome sequence analysis, we further limited de novo variant calls only to those predicted to modify the function or amount of protein [missense (PolyPhen2 probably/possibly damaging), variants in conserved splicing regions (variant at the exon-intron boundary within 3 bases into an exon or 8 bases into the intron), nonsense, or indels]. Given the high rate of false positives when the variant allele fractions were <30%, we only evaluated known pathogenic variants and any variant in *ATP1A3* with variant allele fractions <30% to consider the possibility of mosaicism for the gene-negative AHC cases. The list of de novo variants is provided in Supplementary Table 2.

We also compiled rare hemizygous and homozygous genotypes from each of the probands using the following criteria: (1) homozygous or hemizygous genotype in the proband with at least 10-fold coverage; (2) exclude genotypes at variants sites with MAF <0.5% in any population subset and homozygous and/or hemizygous genotype frequency >0 among internal controls or any population in EVS, Exome Aggregate Consortium (ExAC release 0.3), and gnomAD browser (v2.1.1); (3) exclude variants with a GATK RMSMappingQuality score of <40 and a GQ score of <20 in the proband; (4) exclude genotypes in the proband with a variant allele fraction of <80%; (5) exclude variants in RepeatMasker regions; (6) in cases where sequencing of unaffected was performed, both parents were required to have 10-fold coverage at the variant site and the mother was required to be heterozygous for hemizygous candidates in the proband, and both mother and father were required to be heterozygous for homozygous candidates in the proband parents were required to have a heterozygous genotype; and (7), for exome sequence analysis, we further limited homozygous and hemizygous variant calls only to those predicted to modify the function or amount of protein [missense (PolyPhen2 probably/possibly damaging), variants in conserved splicing regions (variant at the exon-intron boundary within 3 bases into an exon or 8 bases into the intron), nonsense, or indels]. The list of candidate homozygous and hemizygous genotypes in the probands is provided in Supplementary Table 2.

Table 1. Study cohort.

family ID	subject ID	phenotype	gender	trio	affected family members	genomic analysis	% of full *ATP1A3* gene[a]	% of protein-coding regions of *ATP1A3*[b]
ahcbh	ahcdukeepi4538bh1	atypical	female	yes	no	exome/targeted sequencing	97.44	97.8
ahcbv	ahcdukeepi4542bv1	atypical	female	no, proband only	no	exome/targeted sequencing	99.82	98.7
ahcaw	ahcdukeepi2937aw1	atypical	female	yes	no	exome/targeted sequencing	98	96.1
ahcn	ahcdukeepi3911n4	atypical	female	yes	yes, affected half-sibling (same mother)	exome/targeted sequencing	95.54	98.7
ahcbj	ahcdukeepi4512bj1	atypical	female	yes	no	exome/targeted sequencing	91.33	98.2
ahcbw	ahcdukeepi4542bw1	atypical	male	no, proband only	no	exome/targeted sequencing	99.41	98.7
ahcao	ahcdukeepi3905ao1	atypical	male	yes	no	exome/targeted sequencing	94.83	96.6
ahcbz	ahc14A84bz1	typical	female	yes	no	exome/targeted sequencing	98.96	98.6
ahcbi	ahcdukeepi4529bi1	typical	female	yes	no	exome/targeted sequencing	97.85	98.7
ahcbd	ahcdukeepi4588bd1	typical	female	yes	no	exome/targeted sequencing	97.66	98.7
ahcx	ahcdukeepi3902x1	typical	female	no, proband only	no	exome/targeted sequencing	96.98	98.5
ahcbb	ahcdukeepi3937bb1	typical	female	no, proband only	no	exome/targeted sequencing	97.84	98.3
ahcbs	ahcdukeepi4542bs1	typical	female	no, proband only	no	exome/targeted sequencing	99.99	98.7
ahcaa	ahc13A3478aa1ex	typical	female	yes	no	exome/targeted sequencing	99.74	98.7
ahcby	ahc150010by1	typical	female	yes	no	exome/targeted sequencing	99.04	98.2
dukeepi4547	ahcdukeepi4547bf4	typical	male	yes	three additional affected full sublings	exome/targeted sequencing	97.22	98.7
ahcbo	ahcdukeepi4524bo1	typical	male	yes	no	exome/targeted sequencing	97.68	97.9
ahcbc	ahcdukeepi4593bc1	typical	male	yes	no	exome/targeted sequencing	97.08	98.7
ahcad	ahc13A1635ad1ex	typical	male	yes	no	exome/targeted sequencing	99.5	98.7
ahcab	ahc13A2107ab1ex	typical	male	yes	no	exome/targeted sequencing	99.03	98.2
ahcag	ahc13A581ag1ex	typical	male	yes	no	exome/targeted sequencing	98.77	98.7
ahcah	ahc157184ah1ex	typical	male	no, proband only	no	exome/targeted sequencing	99.29	98.7

Table 1. continued

family ID	subject ID	phenotype	gender	trio	affected family members	genomic analysis	% of full *ATP1A3* gene[a]	% of protein-coding regions of *ATP1A3*[b]
ahcbe	ahcdukeepi4580be1	atypical	female	yes	one additional affected full subling	exome	NA	98.7
ahcav	ahcit01ap1	typical	female	yes	no	exome	NA	98.7
ahci	ahc149666i1	typical	female	yes	no	exome	NA	98.7
ahck	ahcahc2k1	typical	female	yes	no	exome	NA	98.7
ahcac[c]	ahc13A2344ac1	typical	male	no, proband only	no	targeted sequencing of *SCN2A*	NA	NA
IT06[c]	IT06	typical	female	no, proband only	no	exome	NA	NA

NA not applicable.
[a]Sequenced at least 10-fold in proband.
[b]Sequenced at least 10-fold in proband (exome and targeted sequencing sequencing).
[c]Cases identified after original sequencing study.

Finally, compound heterozygous genotypes from the proband were compiled by first compiling all qualifying heterozygous variant calls that included (1) heterozygous variant call in the proband with at least 10-fold coverage; (2) exclude variants with MAF > 0.5% in the Institute of Genomic Medicine control cohort, and in EVS, Exome Aggregate Consortium (ExAC release 0.3), and gnomAD browser (v2.1.1); (3) exclude variants with a GATK RMSMappingQuality score of <40, QualbyDepth Score <2, quality score <50; (4) exclude variants with a variant allele fraction of <30% and <70%; (5) exclude variants in RepeatMasker regions; and (6), for exome sequence analysis, we further limited candidate de novo variant calls only to those predicted modify the function or amount of protein [missense (PolyPhen2 probably/possibly damaging), variants in conserved splicing regions (variant at the exon-intron boundary within 3 bases into an exon or 8 bases into the intron), nonsense, or indels].

Compound heterozygous genotypes were only evaluated in trios where we could confirm bi-allelic inheritance. The list of compound heterozygotes in the probands is provided in Supplementary Table 2.

Variants were assessed for pathogenicity using the principles outlined by the American College of Medical Genetics (ACMG) [15].

Functional evaluation of *ATP1A2* variants

Human Na^+/K^+-ATPase α2 subunit cDNA was subcloned as previously described [16]. In brief, to distinguish endogenous Na^+/K^+-ATPase activity from that of transfected Na^+/K^+-ATPase, two additional mutations were introduced in the original α2 subunit cDNA to express an ouabain-resistant isoform. Next, FHM2 missense variants p.E332Q and p.M813K were introduced into the ouabain-resistant wild-type α2 subunit construct by site-directed mutagenesis. All constructs were sequence verified.

HeLa cells (5×10^5) were transfected with 1.6 µg plasmid DNA using Lipofectamine 2000 Transfection Reagent in Opti-Mem medium (Invitrogen, Waltham, MA, USA) and cultured in DMEM-containing Glutamax and 10% FCS. Two days after transfection, one-third of the cells were seeded on 10-cm petri dishes and after 5 days of ouabain (1 µM) challenge, colonies were stained with 1% methylene blue in 70% methanol, scanned, and analyzed with ImageJ (NIH and LOCI, University of Wisconsin, WI, USA). Each transfection was performed three times in triplicates. Two days after transfection, two-thirds of the cells were harvested for Western blot analysis as described previously [16]. The α2 subunit-specific polyclonal antibody HERED was used for staining [17]. The blot was incubated with the secondary antibody goat anti-rabbit Alexa FluorTM 680 (Abcam, Cambridge, UK) and scanned, with the Odyssey Imaging System (LI-COR Biosciences, Lincoln, NE, USA).

Functional evaluation of *SCN2A* variants

HEK293T cells stably transfected with the human sodium channel β1 (*SCN1B*) and β2 (*SCN2B*) auxiliary subunits (HEK-beta cells) were maintained in Dulbecco's modified Eagle's medium (GIBCO/Invitrogen, San Diego, CA, USA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA, USA), 2 mM L-glutamine, 50 units/mL penicillin, and 50 µg/mL streptomycin at 37 °C in 5% CO2. For automated electrophysiology experiments, full-length WT or variant *SCN2A* ($Na_V1.2$) cDNA was electroporated into HEK-beta cells using the MaxCyte STX electroporation system (MaxCyte Inc., Gaithersburg, MD, USA). Automated patch clamp recording was performed using the Nanion Sycropatch 768PE platform (Nanion Technologies, Munich, Germany) using single-hole low resistance (3–4 MΩ) recording chips. Pulse generation and data collection were performed using PatchControl384 v1.6.6 and DataControl384 v1.6.0 software (Nanion Technologies, Munich, Germany). Whole-cell currents were acquired at 10 kHz, series resistance was compensated 80%, and leak currents were subtracted using P/4 subtraction. The external solution contained (in mM): 140 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1 HEPES, 5 glucose, with the final pH adjusted to 7.4 with NaOH, and osmolality adjusted to

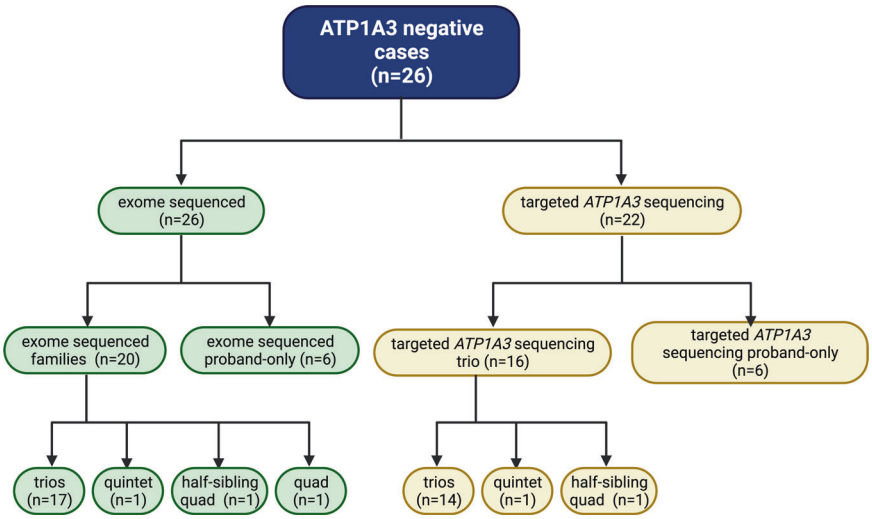

Fig. 1 Cohort overview. Cohort breakdown for individuals undergoing exome ($n = 26$, green shading) and targeted ($n = 22$, yellow shading) sequencing of *ATP1A3*. Exome and panel sequenced cohorts are further subdivided into cases where familial samples were available and proband-only cases.

300 mOsm/kg/L with sucrose. The composition of the internal solution was (in mM): 110 CsF, 10 CsCl, 10 NaCl, 20 EGTA, 10 HEPES, with the final pH adjusted to 7.2 with CsOH, and osmolality adjusted to 300 mOsm/kg/L with sucrose. High-resistance seals were obtained by addition of seal enhancer solutions, which was comprised of (in mM): 80 NaCl, 3 KCl, 35 $CaCl_2$, 10 $MgCl_2$, 10 HEPES, with the final pH adjusted to 7.4 with NaOH. Prior to recording, cells were washed twice with external solution, and the final concentrations of $CaCl_2$ and $MgCl_2$ were 3 mM and 2 mM, respectively. Biophysical data were collected only from cells with currents larger than −200 pA. Stringent criteria were set to select cells included in the final analysis seal resistance ≥200 MΩ, access resistance ≤20 MΩ, capacitance ≥2 pF, and sodium reversal potential between 45 and 85 mV. Voltage control was assessed from conductance-voltage (GV) curves and cells were included in the final analysis if two adjacent points on the GV curve showed no more than a 7-fold increase. Unless otherwise noted, all chemicals were obtained from SigmaAldrich (St. Louis, MO, USA). Data were analyzed and plotted using a combination of DataControl384 v1.6.0 (Nanion Technologies, Munich, Germany), Clampfit 10.4 (Molecular Devices, San Jose, CA, USA), Microsoft Excel (Microsoft Office 2019, Remond, WA, USA), and GraphPad Prism (GraphPad Software, San Diego, CA, USA). Whole-cell currents were normalized to membrane capacitance, and data are expressed as mean ± SEM unless otherwise noted. One-way ANOVA with Dunn's *post hoc* test was used for statistical comparison, and the threshold for statistical significance was $P \leq 0.05$.

RESULTS

Analysis of *ATP1A3*

The protein-coding sequencing from both targeted and exome sequencing of *ATP1A3* in the 26 probands was nearly complete with 98% of protein-coding exons sequenced at least 10-fold on average (min. 96%) (Table 1). Across all of the 22 individuals undergoing targeted sequencing, we identified no mosaic or germline de novo variants in protein-coding or non-coding regions in leukocyte-derived DNA. We also found no homozygous or compound heterozygous *ATP1A3* variants in any of the cases.

Exome sequence analysis

De novo variants. A total of 31 protein-coding putatively functional de novo variants were identified in the 20 trios who underwent exome sequencing. Only one gene, *RHOBTB2*, had de novo variants in multiple individuals with three individuals having three different missense variants. Observing three de novo variants in three different individuals accounting for gene size, sequence mutability is highly unlikely to occur by chance (FitDNM, $P = 1.3 \times 10^{-11}$) [18]. Even correcting for all of the 18 K genes using a Bonferroni correction, the enrichment is still significant.

American College of Medical Genetics and Genomics (ACMG) diagnostic analysis. In addition to the three likely pathogenic variants in *RHOBTB2*, we also identified pathogenic or likely pathogenic de novo variants in additional probands in *ATP1A2* ($n = 1$), *ANK3* ($n = 1$), *CHD2* ($n = 1$), and *SCN2A* ($n = 1$). The de novo variant in *CHD2* was identified in a family with two affected children, however, the other affected child did not carry the *CHD2* variant. Clinically, the *CHD2* variant was deemed to be pathogenic and partially contributing to the phenotype of the proband. Overall, no pathogenic or likely pathogenic compound heterozygous variant sets or homozygous or newly hemizygous variants were identified.

One variant of unknown significance was identified in the exome sequence data, a stop-gained variant *RHOBTB2* that was inherited from an unaffected father (Table 2, Supplementary Table 2).

Phenotyping and functional characterization

RHOBTB2: In total, we identified three likely pathogenic variants and one variant of unknown significance (VOUS) in *RHOBTB2* (Table 2). Two of the cases with a genetic diagnosis presented with typical AHC (ahc13A581ag1ex and ahcahc2k1) while one presented atypically lacking obvious bouts of hemiplegia or quadriplegia (ahcdukeepi4538bh1). The case with the stop gained VOUS (ahcdukeepi4542bv1) also presented atypically with unclear information regarding bouts of hemiplegia or quadriplegia (Supplementary Table 1).

ATP1A2: Among the exome-sequenced cases, we identified three rare variants in *ATP1A2*, including one likely pathogenic de novo variant, one likely pathogenic variant with unknown inheritance, and one likely pathogenic splice site variant in *ATP1A2* inherited from a father who is diagnosed with hemiplegic migraine. Only the likely pathogenic variant with unknown inheritance presented with an atypical presentation with no reports of quadriplegia or relief from hemiplegic attacks upon sleeping (ahcdukeepi4542bw1, Supplementary Table 1). None of the three probands were deemed

Table 2. Candidate genetic diagnoses in *ATP1A3*-negative AHC cohort.

Sample ID	inheritance	Variant ID (chr-position-ref-alt, hg19)	Variant Type	GeneName	Transcript	HGVS_c	HGVS_p	ACMG classification
ahc149666i1	de novo	10-62023779-C-T	snv	*ANK3*	ENST00000280772	c.514-1 G > A	NA	likely pathogenic
ahc14A84bz1	de novo	1-160097587-G-C	snv	*ATP1A2*	ENST00000361216	c.994 G > C	p.E332Q	likely pathogenic
ahcdukeepi4542bw1[a]	inheritance unknown	1-160105782-T-A	snv	*ATP1A2*	ENST00000361216	c.2438 T > A	p.M813K	likely pathogenic
ahcdukeepi4524bo1[a]	inherited from father with hemiplegic migraine	1-160097615-G-A	snv	*ATP1A2*	ENST00000361216	c.1017+5 G > A	NA	likely pathogenic
ahcdukeepi4580be1	de novo	15-93540315-GAA-G	indel	*CHD2*	ENST00000394196	c.3722_3723delAA	p.E1241fs	likely pathogenic
ahc13A581ag1ex	de novo	8-22864414-C-T	snv	*RHOBTB2*	ENST00000251822	c.656 C > T	p.S219F	likely pathogenic
ahcahc2k1	de novo	8-22861984-G-A	snv	*RHOBTB2*	ENST00000251822	c.37 G > A	p.E13K	likely pathogenic
ahcdukeepi4538bh1	de novo	8-22865140-G-A	snv	*RHOBTB2*	ENST00000251822	c.1382 G > A	p.R461H	pathogenic
ahcdukeepi4542bv1	inherited from unaffected father[c]	8-22862900-C-T	snv	*RHOBTB2*	ENST00000251822	c.208 C > T	p.R70*	VOUS
ahcdukeepi4593bc1	de novo	2-166237628-CAGA-C	indel	*SCN2A*	ENST00000283256	c.4477_4479delGAA	p.E1493del	pathogenic
IT06[b]	de novo	2-166245267-T-G	snv	*SCN2A*	ENST00000283256	c.4951 T > G	p.F1651V	likely pathogenic
ahc13A2344ac1[b]	unknown	2-166243356-A-G	snv	*SCN2A*	ENST00000283256	c.4652 A > G	p.E1551G	likely pathogenic

Indel insertion -deletion, *snv* single nucleotide variation, *NA* non applicable.
[a]Patient previously reported in Moya-Mendez et al (2021) [19] .
[b]Variants identified in separate studies (see Materials and Methods).
[c]Inherited determined from clinical sequencing; VOUS variant of unknown significant.

Fig. 2 Ouabain-survival assay in transfected HeLa cells. A Western blot analysis of HeLa cells transfected with wild-type (WT) or *ATP1A2* variant cDNA. (anti-HERED antibody). **B** Ouabain sensitivity as determined by cell survival of cells transfected with either wild-type or *ATP1A2* cDNA variants. Bars represent cell survival after 5 days of ouabain treatment ($n = 3$). Error bars show standard error of the mean. WT, p.E332Q, and p.M813K, studied variants. The glutamate residue at position 332 is part of the ion-binding pocket of the Na^+/K^+-ATPase [30]. Structural effects of replacing glutamic acid with glutamine (E332Q) can lead to a minor conformational shift of the amino acid, disrupting the binding pocket resulting in the observed decrease in cell survival. The methionine residue at position 813 is located in the sixth transmembrane domain, close to cation-binding amino acid aspartate 808. Replacement of this amino acid with the positively charged lysine most likely disturbs the binding pocket in such a way that in our assay cell survival is decreased to zero.

clinically to have hemiplegic migraine (Supplementary Table 1). We note that two of the individuals with likely pathogenic *ATP1A2* variants (ahcdukeepi4542bw1 and ahcdukeepi4524bo1) were reported in a previous publication [19] and reported to have an epileptic encephalopathy in addition to having clinical presentations consistent with AHC. In vitro functional evaluation of p.E332Q and p.M813K demonstrated that the expression levels were comparable or higher than that of the wild-type, whereas cell survival was decreased to 14% and 0%, respectively (Fig. 2). This indicates that both variants have functional consequences on sodium–potassium pump functioning and can be considered pathogenic. Consistent with these in vitro functional findings, a previous publication reported AHC-like phenotypes in an M813K mouse model [19].

SCN2A: In addition to the identification of the in-frame de novo *SCN2A* indel (p.E1493del) in one of the patients included in this study, we had identified (likely) pathogenic rare missense variants (p.E1551G, p.F1651V) in two additional cases that met the AHC criteria prior to the initiation of this study. One of the newly identified *SCN2A* variants was de novo and the other was of unknown inheritance (parental samples were not available). In vitro functional evaluation of all three variants demonstrated varying patterns of dysfunction (Fig. 2; Supplemental Table 3). The de novo *SCN2A* indel variant exhibited current density that was not significantly different from background (endogenous) indicating a complete loss-of-function. By contrast, the two missense variants were functional but had significantly altered properties including depolarized voltage-dependence of steady-state inactivation, slower time course of fast inactivation, significantly enhanced channel activation during a slowly depolarizing voltage ramp (p.E1551G only), slower recovery from inactivation and greater loss of activity with repeated depolarizations. These functional features support the pathogenicity of the *SCN2A* variants.

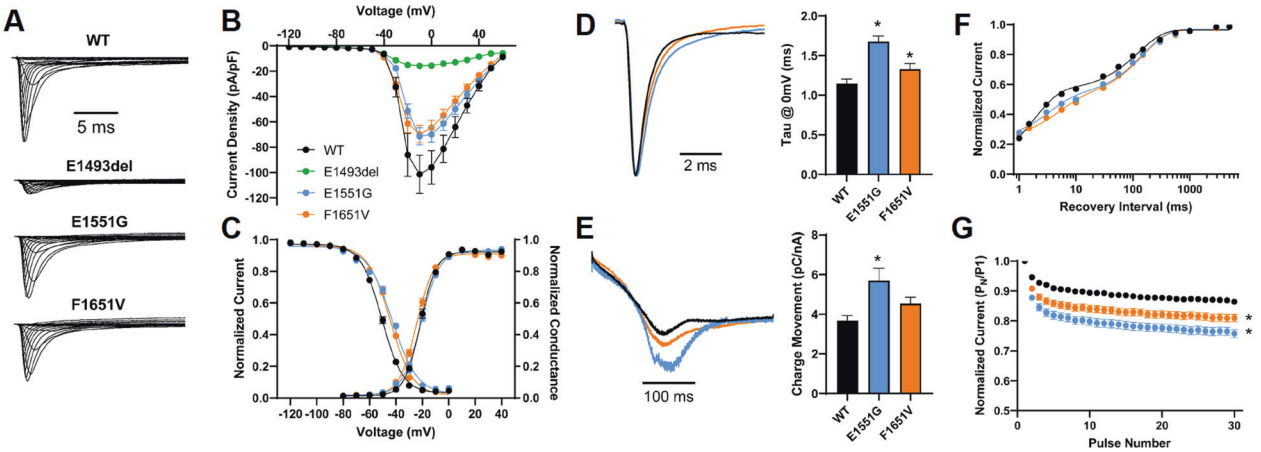

Fig. 3 Functional properties of AHC2-associated *SCN2A* variants. A Average whole-cell sodium currents normalized to peak current of the wild-type (WT) channel. **B** Current-voltage relationships. **C** Voltage-dependence of activation and inactivation. **D** Average normalized whole-cell sodium currents elicited at 0 mV, and summary data showing time-constant of fast inactivation. **E** Average whole-cell ramp currents normalized to wild-type current and summary data showing charge movement elicited by the voltage ramp. **F** Time course of recovery from inactivation. **G** Frequency dependent run down of peak current at 20 Hz. All data are expressed as mean ± SEM with 28 to 120 cells per group for panels **A**–**C**, **F**, **G**, and 12-42 cells per group for (**D** and **E**). Color coding is shown in (**B**). Asterisks indicate $P < 0.05$ for differences between variant and wild-type SCN2A.

DISCUSSION

In this study, we sought to determine the genetic cause of 26 unrelated probands with AHC or an AHC-like phenotype with no previously identified molecular diagnosis. Comprehensive (intronic, exonic, and 1-kb flanking regions) and exome sequencing of the *ATP1A3* gene found no evidence of overlooked *ATP1A3* variants. However, likely genetic diagnoses were identified in 11 probands (42%) in five genes, including *RHOBTB2* ($n = 3$), *ATP1A2* ($n = 3$), *ANK3* ($n = 1$), *SCN2A* ($n = 3$), and *CHD2* ($n = 1$). Each of these genes has been previously associated with neurodevelopmental (or neurological) disorders [16, 20], and two (*RHOBTB2* and *ATP1A2*) have been specifically implicated in AHC or AHC-like presentations [9, 11, 21]. These observations are consistent with several isolated case reports of AHC or AHC-like presentations in individuals with likely pathogenic variants in neurodevelopmental disorder genes not classified as AHC genes (*cf*. Panagiotakaki et al. 2023) [20].

ATP1A2 was first associated in 2004 when a rare variant was found to co-segregate in a large multiplex family with AHC and familial hemiplegic migraine [8]. Mutations in *ATP1A2* are, however, most commonly known to cause hemiplegic migraine type 2 [22], a rare autosomal dominant severe form of migraine with aura [23]. In hemiplegic patients, the aura consists of transient motor weakness varying from mild paresis to hemiplegia. There is overlap in the phenotypic spectrum between hemiplegic migraine and AHC [8, 24] based on clinical similarities of the attacks and the paroxysmal nature of both disorders. A distinction can be made based on choreoathetosis, dystonic posturing, and a progressive course associated with intellectual disability [2, 21, 25]. Our genetic findings, coupled with functional analyses supporting pathogenicity, further solidify the association of *ATP1A2* in AHC.

RHOBTB2 likewise was previously associated with AHC and AHC-like presentations [11]. In that study [11], 11 affected patients were described all with rare heterozygous missense variants in exon 9; in nine patients it was possible to assess the parent of origin and it was found that the variants had originated de novo. Two out of three of the likely pathogenic variants in *RHOBTB2* identified in this study were located in exon 9 (exon 5 of the protein-coding sequence) (Table 2). The majority of our cases had presentations that met the criteria of AHC or had significant phenotypic overlap. An additional case of a rare de novo variant in *RHOBTB2* was later reported in another case with an AHC-like presentation [10].

In addition to identifying variants in known genes (*ATP1A2* and *RHOBTB2*), we also identified a rare de novo in-frame indel variant in *SCN2A*. While de novo *SCN2A* mutations have been reported extensively in neurodevelopmental disorders that sometimes include movement disorders including dystonia and episodic ataxia [2, 26, 27], we are the first to report a case that meets the AHC diagnostic criteria. Follow-up investigations identified two additional *ATP1A3*-negative typical AHC cases who were found to have a likely pathogenic *SCN2A* de novo variant in a parallel sequencing initiative. Even though the parent of origin of the other *SCN2A* variant could not be ascertained, functional data generated in this study support the variant's pathogenicity (Fig. 3).

Despite comprehensive exome sequencing, 65% ($n = 17$) of the cases studied remain genetically unexplained. These cases may be caused by variants not captured by our analyses, including non-coding variants, variants not detectable with short-read next-generation sequencing technology (complex structural rearrangements, short-tandem repeat, and copy number variants), poly- or oligogenic genetic architecture, post-zygotic events undetectable in blood, or possibly phenocopies. Future research is needed to investigate other sources of genetic variation that may explain the outstanding genetically unexplained AHC cases.

Our findings collectively suggest that some individuals with AHC or AHC-like presentations without an *ATP1A3* variant are due to genetic variants in a small number of known neurodevelopmental disease genes that have clinical presentations that have some features associated with AHC. Given the highly variable phenotypic presentation for *ATP1A3* [6, 7, 25, 28, 29] and the genetic heterogeneity observed in this study, thorough investigation of *ATP1A3* and other neurodevelopment disease genes in the clinical genetic diagnostic workup in individuals with AHC or clinical features associated with AHC is warranted.

DATA AVAILABILITY

The individuals did not consent to controlled release of the data into dbGAP or SRA, however, the raw data analyzed in this study are available from the corresponding author on reasonable request.

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AUTHOR CONTRIBUTIONS

EP, FDT, MAM, FG, AMJMvdM and ELH conceived and designed the study. EP, FDT, MAM, AVEH, LSV, FG, AMJMvdM and ELH drafted or revised the manuscript. EP, FDT, MAM, LSV, SN, AVEH, NMW, FG, AMJMvdM, and ELH generated and interpreted sequence data. CHT, ALG and JK generated and interpreted the functional data. EP, MAM, SN, GL, EA, AN, LDP, NMW, EDG, ALP, VDP, AL, MCN, AA and RV provided patient samples, phenoptyic data, and interpreted interpreted genotype-phenotype correlations. All authors reviewed and approved the final version and agreed to be accountable for all aspects or the work.

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COMPETING INTERESTS

ALG serves on a scientific advisory board for Tevard Biosciences, and consults for Praxis Precision Medicines. ALG receives grant funding from Tevard Biosciences, Praxis Precision Medicines, and Neurocrine Biosciences for unrelated work. AMJMvdM received funding from Schedule 1 Therapeutics and Praxis Precision Medicines for unrelated work. The other authors declare no conflicts of interest.

ETHICAL APPROVAL

All subjects were consented to participate in this research study through protocols approved by local ethics boards.

ADDITIONAL INFORMATION

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