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

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Author Contributions MAM, SMS, and DBG jointly supervised this research. ELH, YH, SMS, MAM, DBG conceived and designed the study. Genetic data were generated and analyzed by ELH, KJS, YH, FG, SN, BdV, FDT, CH, LBJ, KVS, CG, LL, GN, AA, and AMJMvdM. Patient DNA samples and phenotypic information of the AHC patients were collected, compiled, and analyzed by KJS, FG, SN, NMW, BdV, FDT, BF, SH, EP, MTS, TMN, LV, SPR, KJM, KS, LJP, JH, MDF, AMB, GKH, CMW, DW, BJL, PU, MDK, IES, GN, AA, SMS, MAM, the European AHC Genetics Consortium, IBAHC Consortium, and the ENRAH for SME Consortium. ELH, AMJMvdM, SMS, MAM, and DBG wrote the paper. All authors reviewed the compiled manuscript.

URLs Ensembl database, www.ensembl.org
Human Gene Mutation Database (HGMD), www.hgmd.org
I.B.AHC Biobank and Clinical Registry for Alternating Hemiplegia, http://en.ibahc.org
Image J software, http://rsbweb.nih.gov/ij/
NHLBI Exome Sequencing Project (ESP) Exome Variant Server, http://snp.gs.washington.edu/EVS
SequenceVariantAnalyzer (SVA), http://www.svaproject.org

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Abstract

Alternating hemiplegia of childhood (AHC) is a rare, severe neurodevelopmental syndrome characterized by recurrent hemiplegic episodes and distinct neurologic manifestations. AHC is usually a sporadic disorder with unknown etiology. Using exome sequencing of seven patients with AHC, and their unaffected parents, we identified *de novo* nonsynonymous mutations in *ATP1A3* in all seven AHC patients. Subsequent sequence analysis of *ATP1A3* in 98 additional patients revealed that 78% of AHC cases have a likely causal *ATP1A3* mutation, including one inherited mutation in a familial case of AHC. Remarkably, six *ATP1A3* mutations explain the majority of patients, including one observed in 36 patients. Unlike *ATP1A3* mutations that cause rapid-onset-dystonia-parkinsonism, AHC-causing mutations revealed consistent reductions in ATPase activity without effects on 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 this gene.

Alternating hemiplegia of childhood (AHC) was first characterized as a distinct syndrome in 1971 with a report describing eight patients with episodes of intermittent hemiplegia on alternating sides of the body, developmental delay, dystonia, and choreoathetosis beginning in infancy[1]. Since that time, specific diagnostic criteria have more clearly defined the classic paroxysmal and interictal neurologic manifestations associated with this disease[2-6]. AHC is estimated to affect approximately one in one million individuals[7], with most cases occurring sporadically[5,8-10]. While 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*[11,12]. To date, no cases of sporadic AHC have been attributed to *ATP1A2* mutations.

In this study, we used next-generation sequencing (NGS) to exome or whole-genome sequence ten AHC probands and their unaffected parents where possible. We identified and confirmed rare (MAF <0.01%) mutations in *ATP1A3* in eight of the 10 probands; for all seven patients where parental DNA was available we could demonstrate that the mutations had occurred *de novo*. The *ATP1A3* mutations included five distinct nonsynonymous mutations, one of which was found in four AHC patients (Supplementary Table 1). *ATP1A3* was then further interrogated in the two unexplained AHC probands for structural variants in the whole genome sequence data, and for overlooked single nucleotide and insertion-deletion variants by Sanger sequencing of the protein coding exons; 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 AHC patients, provides definitive genetic evidence that mutations in *ATP1A3* cause sporadic AHC.

We then Sanger-sequenced the protein-coding exons of *ATP1A3* in an additional cohort of 95 AHC patients. In these 95 subjects, we identified rare (MAF <0.01%) *ATP1A3* mutations in 74 patients (Table 1), all of which were found to be *de novo* in the 59 sporadic AHC patients with parental DNA available. Including samples sequenced with NGS, we, in total,

identified 18 different *ATP1A3* mutations in 82 out of 105 (78%) patients studied. The majority of these mutations fell in or near transmembrane domains of ATP1A3 (Fig. 1). Six of the mutations were identified in multiple AHC cases, including D801N and E815K that were identified in 36 (34%) patients and 19 (18%) patients, respectively (Table 1). One of the 95 AHC patients evaluated was a case of autosomal dominant alternating hemiplegia, first described in 1992[8]. In this familial case of AHC, we identified a rare *ATP1A3* mutation (I274N) in the cytoplasmic domain that co-segregates with the AHC phenotype (Fig. 2).

Thirteen of the 17 *ATP1A3* mutations seen in sporadic AHC cases were confirmed to be *de novo* (Supplementary Table 1). We also observed, however, 15 sporadic patients with rare (MAF <0.01%) *ATP1A3* variants where parents were not available. This raises the possibility that some of these are inherited benign polymorphisms. While this is unlikely given the rarity of functional variants in *ATP1A3*, we can conservatively estimate the number of patients with pathogenic *ATP1A3* mutations by only considering those mutations observed as *de novo* in at least one patient as pathogenic. Under this assumption, 11 of the 15 patients have a pathogenic *ATP1A3* mutation. We can therefore conclude that at least 74% of sporadic patients with typical presentation of AHC studied here harbor disease-causing mutations in *ATP1A3*.

ATP1A3 encodes an alpha-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 AHC patients. Two AHC mutations (D801N and I274N), however, affect the same amino acids as the DYT12 mutations.

To better understand how *ATP1A3* mutations cause two clinically distinct disorders, the functional consequences of the five mutations identified in the NGS screens of AHC patients and ten mutations that cause DYT12 were studied *in vitro*. All fifteen mutations were introduced in expression constructs and assessed for *ATP1A3* expression and function. None of the mutations was found to affect *ATP1A3* mRNA expression (Supplementary Fig. 1). However, seven out of ten mutations that cause DYT12 reduced ATP1A3 protein expression to undetectable levels, while none of the AHC-causing mutations reduced protein expression compared to wild-type (Fig. 3 and Supplementary Fig. 2). Despite different effects on protein expression both the mutations that cause AHC and those that cause DYT12 reduced ATPase activity *in vitro* by 54-90% (Fig. 3). We note that the previous study of ATP1A3 protein expression in DYT12 reported that D801Y attenuates protein expression and I274T does not affect protein expression[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, both studies show that DYT12 mutations typically reduce ATP1A3 protein expression, whereas in our study no AHC mutation reduces protein levels. These data suggest that *ATP1A3* mutations causing DYT12 do so through hypomorphic effects on the Na^+/K^+-ATPase pump, while AHC-causing mutations modulate the activity of the pump. The reason for this inference is that if hypomorphic mutations could cause AHC we would expect some of the mutations to reduce protein level, and that the mutations would be distributed through the protein rather than concentrated in transmembrane domains. Further supporting the hypothesis, evaluation of the crystal structure of the Na^+/K^+-ATPase[19] predicts that a change from aspartic acid at position 801 to asparagine (i.e. D801N) in AHC will prevent the binding of potassium ions. One possible exception to this pattern is the *de novo* splice-site mutation in an AHC patient that may result in protein elimination by a frame-shift, although it could result in a protein with altered activity.

As noted previously, six of the AHC mutations result in the same amino acid substitution in multiple patients. A recurrent mutation in *FGFR3* was previously reported to cause the majority of cases of achondroplasia[20], suggesting the presence of hypermutable sequence in that gene. In AHC, the recurrence of *de novo* mutations may be due to 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 a simple analysis. We observe 13 sites that carry *de novo* mutations in *ATP1A3*. Under the null hypothesis that the mutation rate is equal amongst these sites, the chance that any single site would have 36 or more mutations (as observed for D801N) out of the total 77 observations (patients studied with a "pathogenic" mutation) is low ($P < 0.0001$). Furthermore, three of six of these sites recurrently mutated in AHC are G>A substitutions occurring at hypermutable methylated CpG-dinucleotide sequences[21] (D801N, E815K, G947R). It also appears, however, that only a specific subset of *ATP1A3* mutations produce AHC since nearly all identified AHC mutations fall in or near transmembrane domains of the protein, while the DYT12 mutations are more evenly distributed (Fig. 1). Collectively, this suggests that the observed patterns of AHC-causing mutations across *ATP1A3* results from both hypermutable sequence and that only a small set of specific mutations can cause AHC. This is consistent with the functional analyses suggesting that AHC mutations may have specific effects on protein function as opposed to simply reducing activity. While not evaluated in this study, functional evaluation of the inherited *ATP1A3* mutation (I124N) may reveal distinct effects on the activity of the Na^+/K^+-ATPase that may help explain the atypical familial AHC phenotype.

We also evaluated whether patients with and without *ATP1A3* mutations have different clinical presentations. *Myshkin* mice that are heterozygous for missense *Atp1a3* mutation (I801N) that inactivates Na^+/K^+-ATPase are predisposed to seizure activity that is rescued by replacement with functional *Atp1a3*[22]. We therefore first evaluated whether patients with *ATP1A3* mutations were more likely to have seizures than those without, and found a minor but significant effect (54% versus 29% respectively, $P = 0.01$, binomial probability calculation). For one patient cohort studied here (n = 30) with consistent phenotyping[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. More detailed investigations are needed to characterize the phenotypic spectrum associated with *ATP1A3* mutations, and to compare phenotypes among patients with different *ATP1A3* mutations.

In conclusion, mutations in *ATP1A3* likely account for at least 74% of patients with a diagnosis of typical sporadic AHC. Since the present study only assessed 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 patients, 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. Having the ability to test the functional consequences of two groups of mutations that lead to clinically-distinct phenotypes offers unique insight into the pathophysiologic processes unique to each disease and will likely facilitate drug discovery for these and related conditions.

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, the Genetics of Epilepsy study at University College London, and at the University of Utah. Blood samples for DNA extraction were

collected from each affected child and from unaffected parents when possible. Patients comprising 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 = 1), 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 consented based on the standards set forth by the ethics boards at the patient 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 within the Center for Human Genome Variation (Duke University). Sequence data from 484 controls non-enriched for neuropsychiatric diseases that were sequenced as part of other in-house studies (e.g. 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 samples and their parents were sequenced to an average coverage of 90-fold (minimum 65-fold), with at least 95 percent 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 indels 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 (nonsynonymous coding vs. splice site, gene name, transcript, associated GO term, etc)[29]. Identity-by-descent calculations were used to confirm paternity and maternity.

Pictures of aligned NGS fragments covering the *de novo ATP1A3* variants in the seven sequenced AHC trios (parents and affected child) are provided in Supplementary Fig. 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 non-causal. Genotypes present in the patients and absent in the unaffected parents, 484 sequenced controls, and 5400 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-coding exons of *ATP1A3*

Some or all of the protein-coding 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 publically 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 ten common polymorphisms to confirm that samples harboring the same *ATP1A3* mutation and each 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[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 human neuroblastoma A172. Each part of *ATP1A3* cDNA was then subcloned into the pCR-Blunt II-TOPO vector (Invitrogen-Life Technologies, Carlsbad, CA, USA). 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-wildtype vector, and each allele of pcDNA3.1(+)-ATP1A3 vectors were transfected into human epithelial carcinoma cell line HeLa 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).

Western Blotting—Empty pcDNA3.1 (+) vector, pcDNA3.1(+)-ATP1A3-wildtype vector, and each of the pcDNA3.1(+)-ATP1A3-mutant allele vectors were transfected into human epithelial carcinoma cell line (HeLa) and monkey kidney cell line (COS-7) by lipofection. After 48h of transfection, the cell lysates were subjected to SDS–PAGE gel and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA). The membranes were then incubated with anti-human-ATP1A3 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-beta-actin (Cell Signaling Technology, Beverly, MA). Proteins were visualized with the ECL plus western blotting detection system (GE Healthcare, Piscataway, NJ). The effects of the mutations were quantified using Image J 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 with detectable ATP1A3 protein levels in the Western blot assay were assessed in the ATPase assay. COS-7 cells were chosen for the ATPase analysis because HeLa cells do not express *ATP1A2*, an essential subunit of the Na+/K+-ATPase. The lysates were then incubated with 250 μM of ATP, 40mM of NaCl, 25mM of KCl, 3mM of $MgCl_2$ and 1mM EGTA in 37 degree for 1h. 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Web box

David Goldstein and Mohamad Mikati report identification of de novo mutations in ATP1A3 in alternating hemiplegia of childhood, which is a rare neurodevelopmental syndrome characterized by recurrent hemiplegic episodes and distinct neurologic manifestations.

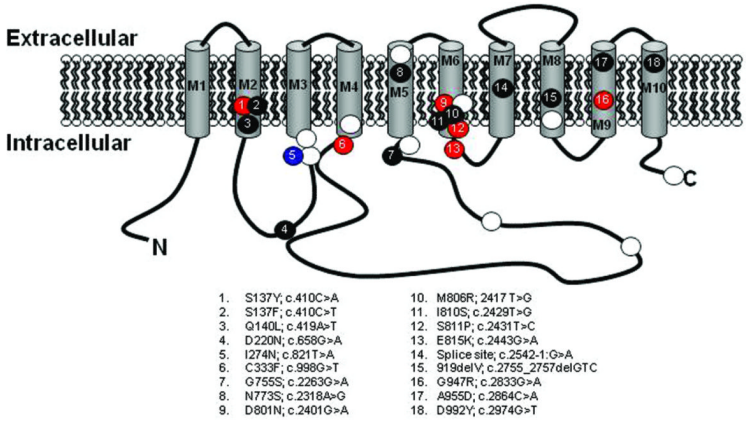

Figure 1. Diagram of the ATP1A3 protein showing positions of AHC-causing and DYT12-causing mutation

AHC-causing mutations seen in a single case, in multiple cases and in a familial case are represented by black, red and blue dots, respectively. The white dots represent DYT12 mutations compiled from the HGMD database[23]. *ATP1A3* mutation coordinates are defined based on UniProt ID P13637[24] and Consensus CDS ID CCDS12594.1[25].

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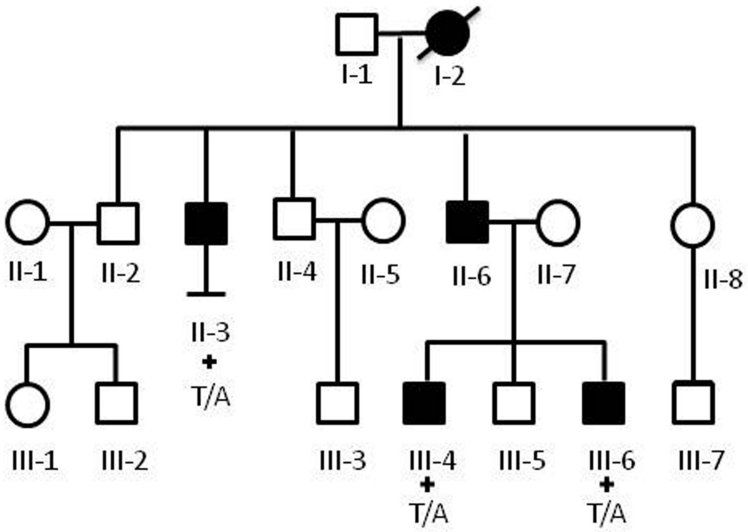

Figure 2. Pedigree of family with autosomal dominant AHC

Black shading indicates affectation status. An *ATP1A3* mutation (c.821T>A;I274N) was identified in patients II-3, III-4, and III-6 where DNA was available (indicated by a plus sign). DNA was unavailable in the father and grandmother of patients III-4 and III-6. Details of the family were reported previously[1]. Phenotypic details of the affected patients are provided in Supplementary Note.

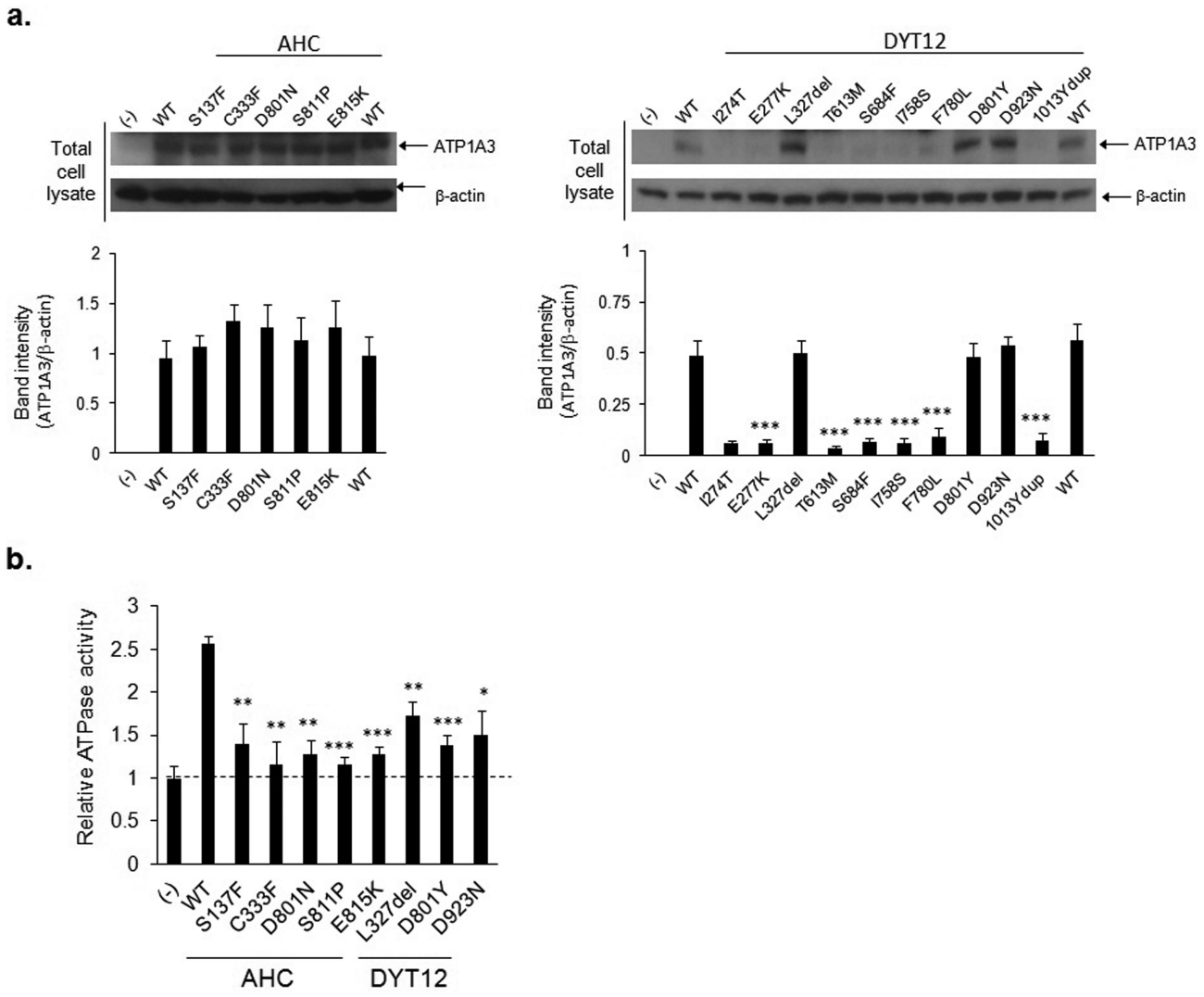

Figure 3. Effects of disease-causing mutations on protein expression and enzyme activity in COS-7 cells

Panel a. shows the effects of AHC-causing mutations (left) to DYT12 (right) on ATP1A3 protein abundance compared to WT. Panel b. compares the ATPase activity of disease-causing mutations (AHC and DYT12, mean ± s.d.) to that of WT.

*P < 0.05, **P < 0.01, ***P < 0.001, Student's t-test (uncorrected for multiple testing), compared to WT

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Table 1

ATP1A3 mutations identified in AHC patients.

ATP1A3 mutation[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	7
A955D	c.2864C>A	1
D992Y	c.2974G>T	1

[a] ATP1A3 mutation coordinates are defined based on UniProt ID P1363724 and Consensus CDS ID CCDS12594.1[25].