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Diagnostic Utility of the ATG9A Ratio in AP-4–Associated Hereditary Spastic Paraplegia

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ABSTRACT

Adaptor protein complex 4–associated hereditary spastic paraplegia (AP-4-HSP), a childhood-onset neurogenetic disorder and frequent mimic of cerebral palsy, is caused by biallelic variants in the adaptor protein complex 4 (AP-4) subunit genes (*AP4B1* [for SPG47], *AP4M1* [for SPG50], *AP4E1* [for SPG51], and *AP4S1* [for SPG52]). Diagnosis is often confounded by variants of uncertain significance. We evaluated the ATG9A ratio, a measure of ATG9A mislocalization in patient-derived fibroblasts, as a functional assay of AP-4 deficiency. In six of eight individuals with suspected AP-4-HSP, the assay demonstrated loss of AP-4 function, establishing pathogenicity of novel variants. These findings support the ATG9A ratio as a clinically useful diagnostic tool for confirming AP-4-HSP and aiding the classification of novel variants.

Trial Registration: ClinicalTrials.gov identifier: NCT06948019, NCT05518188, NCT06692712, NCT04712812

1 | Introduction

Adaptor protein complex 4–associated hereditary spastic paraplegia (AP-4-HSP) is a rare childhood-onset neurogenetic disorder and frequent mimic of cerebral palsy [1, 2], caused by biallelic variants in *AP4B1* (SPG47), *AP4M1* (SPG50), *AP4E1* (SPG51), or *AP4S1* (SPG52) [3]. Clinical diagnosis is often challenging because of nonspecific features and initially only gradual progression, and interpretation of variants of uncertain significance (VUS) remains a major barrier.

AP-4 deficiency disrupts trafficking of the transmembrane protein ATG9A, leading to its accumulation in the trans-Golgi network [4–12]. Prior work has shown that quantifying ATG9A mislocalization in patient-derived fibroblasts can serve as a functional readout of AP-4 loss [4, 13]. With gene replacement therapies for AP-4-HSP in clinical trials (NCT06948019, NCT05518188, NCT06692712), accurate classification of variants has become critical not only for diagnosis but also for determining trial eligibility and guiding treatment decisions. Here, we evaluated the diagnostic utility of the ATG9A translocation assay in individuals with suspected AP-4-HSP carrying VUS,

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with the goal of clarifying variant pathogenicity and supporting clinical diagnoses.

2 | Materials and Methods

2.1 | Patients and Clinical Information

This study was approved by the Institutional Review Board at Boston Children's Hospital (IRB-P00033016). Patients were recruited through the Registry and Natural History Study for Early Onset Hereditary Spastic Paraplegia (NCT04712812). Clinical data were collected using standardized questionnaires, with severity assessed by the Spastic Paraplegia Rating Scale and a four-stage mobility score.

2.2 | Variant Interpretation and Scoring

Variants were standardized to the following transcripts (GRCh38/hg38): *AP4B1* (NM_001253852.3); *AP4E1* (NM_007347.5); *AP4M1* (NM_004722.4). Variants were classified following ACMG guidelines.

2.3 | Reagents and Antibodies

The following reagents were used: Bovine serum albumin (AmericanBIO; #9048-46-8), saponin (Sigma; #47036-50G-F), fetal bovine serum (Cytiva; #SH30396.03HI), DMEM/F-12 (Thermo Fisher Scientific; #10565042), penicillin–streptomycin (10,000 U/mL, #15-140-122; Thermo Fisher Scientific), trypsin EDTA (0.25%, #25200056; Thermo Fisher Scientific) and phosphate-buffered saline (Thermo Fisher Scientific; #10010023). Primary antibodies included anti-ATG9A (1:1000, #ab1083388; Abcam), anti-TGN46 (1:800, AHP500G; Bio-Rad), anti-AP4E1 (1:500, #612019; BD Bioscience), and anti-AP4B1 (1:500, #26081S; Cell Signaling Technology). Fluorescently labeled secondary antibodies (Thermo Fisher Scientific; #A11016, #A11008, #A21235) and Hoechst 33258 were used at 1:2000.

2.4 | Fibroblast Cell Culture and ATG9A Translocation Assay

Fibroblast lines were established from skin punch biopsies and cultured under standard conditions. ATG9A translocation assays were performed as previously described [13]. The ATG9A ratio for each patient was normalized to the ratio of wild-type and loss-of-function control lines processed in parallel (Table 1; Data S1).

2.5 | Western Blot

Western blotting was performed as described previously [4, 13]. Band intensities were normalized to wild-type and loss-of-function control samples run on the same blot (Table 1; Data S1).

3 | Results

Key clinical, genetic, imaging, and functional findings for eight patients are summarized in Table 1 and more detailed case descriptions are provided in Data S1. Western blot and immunocytochemistry data for each case are presented in Figure S1.

Two patients carried VUS in *AP4M1* or *AP4E1* but showed no abnormal ATG9A localization or protein reduction, arguing against AP-4–related disease despite developmental delay and neuroimaging abnormalities. *Patient 1* is a 7-year-old Arab male with intellectual disability, ASD, ADHD, and motor stereotypies, but no other motor symptoms and a homozygous VUS in *AP4M1* [c.405G>T; p.(Gln135His)]. Functional analysis showed no ATG9A mislocalization or AP-4 loss, arguing against AP-4–HSP. *Patient 2* is an 8-year-old male of mixed European background with microcephaly, severe intellectual disability, refractory epilepsy, bulbar dysfunction, and dystonia. WES revealed compound-heterozygous *AP4E1* variants (c.[1276A>C];[3005_3060del], p.[(Ile426Leu)];[(Asn1002Ilefs*12)]). ATG9A ratio and AP4E1 levels were normal, excluding loss of AP-4 function. For both cases, clinical exome sequencing did not identify an alternative diagnosis, and short-read whole genome sequencing is now planned to further investigate a potential underlying genetic cause.

Six patients demonstrated biochemical evidence of AP-4 deficiency (Table 1; Figure S1). Four carried *AP4M1* variants and two carried *AP4B1* variants; all showed elevated ATG9A ratio and protein levels with decreased AP4E1 expression. Clinical features included early hypotonia progressing to spasticity, intellectual disability, seizures, and corpus callosum abnormalities. In *Patient 6*, functional testing established pathogenicity of a VUS in *trans* with a reported pathogenic variant. *Patient 3* is a 15-year-old Irish male with early hypotonia progressing to spasticity, severe intellectual disability, epilepsy, and corpus callosum agenesis, with a homozygous *AP4M1* splice-site variant (c.58+3A>G, p.?). *Patient 4* is a 4-year-old male of mixed European background with delayed motor and speech milestones, progressive spastic gait (Video S1), seizures, and corpus callosum thinning, with compound-heterozygous *AP4M1* variants (c.[1129del];[974+115dup], p.[Leu377PhefsTer67];[?]). *Patient 5* is a 4-year-old German female with microcephaly, progressive lower limb spasticity (Video S2), and compound-heterozygous *AP4M1* variants (c.[1137+1G>T];[1010C>T], p.[?];[(Pro337Leu)]). *Patient 6* is a 1.8-year-old Brazilian/European male with global developmental delay (Video S3), hypotonia, generalized epilepsy, and corpus callosum hypoplasia with a homozygous *AP4M1* variant [c.893T>C; p.(Leu298Pro)]. *Patient 7* is a 5-year-old French-Canadian female with mild global developmental delay, hypotonia, ASD, focal epilepsy with impaired awareness, and lower limb spasticity but normal MRI, with a homozygous *AP4B1* missense variant [c.319C>T; p.(Arg107Trp)]. *Patient 8* is a 3-year-old Dutch male with prenatally-detected ventriculomegaly, hypotonia in infancy evolving to a mixed picture of lower limb spasticity and ataxia (Video S4), and recurrent febrile seizures, with compound-heterozygous *AP4B1* variants (c.[1331G>T];[319C>T], p.[Gly444Val];[Arg107Trp]).

TABLE 1 | Clinical, genetic, imaging, and functional findings in eight patients leading to re-classification of uncertain significance (VUS) in AP-4 subunit genes.

Patient number	1	2	3	4	5	6	7	8
AP-4 subunit	*AP4M1*	*AP4E1*	*AP4M1*	*AP4M1*	*AP4M1*	*AP4M1*	*AP4B1*	*AP4B1*
Allele 1	c.405G>T, p.(Gln135His)	c.3005_3060del, p.(Asn1002Ilefs*12)	c.58+3A>G, p.?	c.1129del, p.(Leu377Phefs*67)	c.1137+1G>T, p.?	c.893T>C, p.(Leu298Pro)	c.319C>T, p.(Arg107Trp)	c.1331G>T, p.(Gly444Val)
Allele 2		c.1276A>C, p.(Ile426Leu)		c.974+115dup, p.?	c.1010C>T, p.(Pro337Leu)			c.319C>T, p.(Arg107Trp)
Zygosity	HOM	cHET	HOM	cHET	cHET	HOM	HOM	cHET
gnomAD 4.1.0 allele frequency	8.673e-6	0	8.156e-6	6.199e-7	9.922e-6	0	2.107e-5	4.337e-6
		1.537e-3		0	1.859e-6			2.107e-5
REVEL	0.37	N/A	N/A	N/A	N/A	0.62	0.54	0.64
		0.21		N/A	0.45			0.54
CADD PHRED	20	N/A	25.8	30	34	28.3	30	25
		24.3		N/A	26.3			30
SpliceAI (type)	N/A	N/A	0.91 (donor loss)	N/A	0.87 (donor loss)	N/A	N/A	N/A
		N/A		0.42 (donor loss)	N/A			N/A
AlphaMissense	0.6046	N/A	N/A	N/A	N/A	0.9859	0.9276	0.8495
		0.1522		N/A	0.5557			0.9276
Sex	Male	Male	Male	Male	Female	Male	Female	Male
Age in years	7.1	8.6	15.6	4.8	4.3	1.8	5.5	3
SPRS	0	43	39	12	14	30	8	19
AP-4-HSP core features								
GDD/ID	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Spasticity	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Hypotonia in infancy	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Postnatal microcephaly	No	Yes	Yes	No	Yes	Yes	N/A	Yes
Epilepsy	No	Yes	Yes	No	No	Yes	Yes	No
Febrile seizure	No	No	Yes	No	No	Yes	Yes	Yes

(Continues)

TABLE 1 | (Continued)

Patient number	1	2	3	4	5	6	7	8
Thin corpus callosum	N/A	Yes	Yes	Yes	N/A	Yes	N/A	Yes
White-matter changes	N/A	Yes	No	Yes	N/A	No	N/A	Yes
Ventriculomegaly	N/A	Yes	Yes	Yes	N/A	Yes	N/A	Yes
Total (out of 9 core features)	1	8	8	6	4	8	5	8
Functional analysis results								
ATG9A ratio (SD)	1.132 ± 0.020 (control range: 1.07–1.15)	1.163 ± 0.011 (control range: 1.13–1.24)	1.651 ± 0.038 (control range: 1.13–1.24)	1.303 ± 0.023 (control range: 1.15–1.19)	1.794 ± 0.071 (control range: 1.13–1.22)	1.830 ± 0.119 (control range: 1.01–1.38)	1.683 ± 0.075 (control range: 1.13–1.22)	1.561 ± 0.030 (control range: 1.24–1.32)
AP4E1 protein level (SD) (fold of control)	1.00 ± 0.037	0.706 ± 0.084	0.458 ± 0.053	0.639 ± 0.111	0.269 ± 0.033	0.597 ± 0.073	0.303 ± 0.068	0.527 ± 0.080
ATG9A protein level (SD) (fold of control)	1.00 ± 0.144	0.740 ± 0.204	3.055 ± 0.423	3.458 ± 1.203	2.719 ± 0.881	2.363 ± 0.449	3.425 ± 0.243	2.587 ± 0.394
ACMG re-classification (including data from functional assay)	LB (PM2, PP1, BS3, BP4)	P (PVS1, PP1, PP4) VUS (PM3, PP1, PP4, BP2, BP4)	LP (PS3, PM3, PP1, PP3, PP4)	P (PVS1, PS3, PM2, PM3, PP1, PP3, PP4) P (PS3, PM2, PM3, PP1, PP4)	P (PVS1, PS3, PM2, PM3, PP1, PP3, PP4) P (PS3, PM2, PM3, PP1, PP3, PP4)	LP (PS3, PM2, PP1, PP3, PP4)	LP (PS3, PM2, PP1, PP3, PP4)	LP (PS3, PM2, PM3, PP1, PP3, PP4) LP (PS3, PM2, PM3, PP1, PP3, PP4)
AP-4-HSP diagnosis	No	No	Yes	Yes	Yes	Yes	Yes	Yes

Note: Tool versions: AlphaMissense, CADD PHRED, and SpliceAI scores were obtained using The Broad Institute SpliceAI Lookup tool on 19/12/2025. *Transcript IDs*: AP4B1 (NM_001253852.3); AP4E1 (NM_007347.5); AP4M1 (NM_004722.4). *Zygosity*: cHET (compound heterozygous); HOM (homozygous); *Variant classifications*: LB, likely benign; LP, likely pathogenic; P, pathogenic; VUS, variant of uncertain significance.
Abbreviations: GDD/ID, global developmental delay/intellectual disability; N/A, not available; SPRS, Spastic Paraplegia Rating Scale.

4 | Discussion

This study highlights the utility of the ATG9A ratio as a functional diagnostic assay for AP-4-HSP. Among eight patients with suspected AP-4-HSP, functional studies confirmed AP-4 deficiency in six and excluded it in two, directly informing the classification of VUS and the accuracy of the clinical diagnosis (Table 1). While our prior work established and validated the ATG9A translocation assay [4, 13]; the present report demonstrates real-world diagnostic utility by applying the assay prospectively to VUS cases encountered in clinical practice and showing how results directly inform ACMG reclassification and clinical decision-making. While the assay is not yet a CLIA-certified diagnostic test, the workflow is mature and feasible in specialized research/diagnostic labs.

The interpretation of VUS remains a major challenge in rare neurogenetic disorders, particularly when phenotypes overlap with cerebral palsy or other developmental conditions. By directly measuring the cellular consequence of AP-4 deficiency—mislocalization of ATG9A—the ATG9A ratio provides a biologically relevant marker that bridges genotype and phenotype [4, 13]. The ATG9A assay provides ACMG criterium PS3 (functional evidence) given (i) prior validation of the assay in AP-4 deficiency and (ii) internal positive/negative controls run in parallel. In combination with other criteria (as applicable to each case) including PM2 (absent/rare in population databases), PP4 (highly specific phenotype with thin corpus callosum/spastic paraparesis), and PM3 (in trans with a pathogenic variant/recessive inheritance), this functional evidence allowed reclassification from VUS to likely pathogenic for several variants. The assay thereby reduces reliance on clinical assessment alone, avoiding misdiagnosis and enabling precise genetic counseling. The complete workflow (from skin biopsy to finalized result) can typically be completed within approximately 4–6 weeks, thereby facilitating timely and accessible diagnostic evaluations.

Importantly, the relevance of correct variant classification now extends beyond diagnosis. With gene replacement therapies for AP-4-HSP [8, 12, 14] in clinical development (NCT06948019, NCT05518188, NCT06692712), eligibility for trials and eventual treatment will depend on confident molecular diagnoses. Functional validation tools such as the ATG9A ratio will be critical to support regulatory submissions, guide patient selection, and ensure that individuals with established AP-4 deficiency are prioritized for emerging therapies.

In summary, our findings establish the ATG9A ratio as a reliable and clinically meaningful assay for confirming AP-4 deficiency, resolving uncertain variants, and preparing the field for the integration of molecular therapies. Incorporating functional diagnostics alongside genomic sequencing may serve as a model for other neurogenetic disorders characterized by frequent VUS and overlapping phenotypes.

Author Contributions

Habibah A. P. Agianda: study design, execution, writing of the first draft, editing of the manuscript. **Hyo-Min Kim:** study design, execution, statistical analysis, writing of the first draft, editing of the manuscript. **Nicole Battaglia:** data collection, editing of the manuscript. **Joshua Rong:** data collection, editing of the manuscript. **Amy Tam:** data collection, editing of the manuscript. **Enrique Gonzalez Saez-Diez:** data collection, editing of the manuscript. **Cornelius F. Boerkoel:** data collection, editing of the manuscript. **Afshin Saffari:** data collection, editing of the manuscript. **Vicente Quiroz:** data collection, editing of the manuscript. **Luca Schierbaum:** data collection, editing of the manuscript. **Zainab Zaman:** data collection, editing of the manuscript. **Katerina Bernardi:** data collection, editing of the manuscript. **Darius Ebrahimi-Fakhari:** study design, execution, statistical analysis, writing of the first draft, editing of the manuscript.

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Conflicts of Interest

C.F.B. is president of Alamya Health. The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** acn370308-sup-0001-DataS1.docx. **Figure S1:** acn370308-sup-0002-FigureS1.pdf. **Videos S1–S4:** acn370308-sup-0003-VideoS1-S4.zip.