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Mutation Screening of *Spastin*, *Atlastin*, and *REEP1* in Hereditary Spastic Paraplegia

Donald S. McCorquodale III[a,‡], **Uzoezi Ozomaro**[a,‡], **Jia Huang**[a], **Gladys Montenegro**[a], **Arielle Kushman**[a], **Luigi Citrigno**[a], **Justin Price**[a], **Fiorella Speziani**[a], **Margaret A. Pericak-Vance**[a], and **Stephan Züchner**[a,*]

[a]John P. Hussman Institute for Human Genomics, University of Miami Miller School of Medicine, Miami, FL, USA

Abstract

Hereditary spastic paraplegia (HSP) comprises a group of clinically and genetically heterogeneous diseases that affect the upper motor neurons and their axonal projections. Over 40 chromosomal loci have been identified for autosomal dominant, recessive, and X-linked HSP. Mutations in the genes *atlastin*, *spastin* and *REEP1* are estimated to account for up to 50% of autosomal dominant HSP and currently guide the molecular diagnosis of HSP. Here we report the mutation screening results of 120 HSP patients from North America for *spastin*, *atlastin, and REEP1*, with the latter one partially reported previously. We identified mutations in 36.7% of all tested HSP patients and describe 20 novel changes in *spastin* and *atlastin*. Our results add to a growing number of HSP disease associated variants and confirm the high prevalence of *atlastin*, *spastin*, and *REEP1* mutations in the HSP patient population.

Keywords

atlastin; Hereditary Spastic Paraplegia; *REEP1*; *spastin*; *REEP1*; *ATL1*; *SPAST*

Introduction

Hereditary spastic paraplegias (HSP) are a clinically and genetically heterogeneous group of neurodegenerative diseases characterized by degeneration of corticospinal tract axons and progressive lower-limb spastic paralysis. HSPs are divided into pure and complicated forms based on additional symptoms such as mental retardation, epilepsy, neurological abnormalities and malformations, or optic atrophy (1,2). HSP is genetically heterogeneous with autosomal dominant, autosomal recessive, and X-linked forms. Genetic studies have revealed as many as 41 different chromosomal HSP loci (3,4). Autosomal dominant HSP represents the most prominent inheritance pattern and mutations in the genes *spastin* (*SPAST*) and *atlastin* (*ATL1*) account for up to 50% of all cases (5). Mutations in *REEP1* (REEP1) are the third most common genetic cause of autosomal dominant HSP (6,7). All other dominant genes (*KIF5A*, *HSP60*, *NIPA1*, *KIAA1096*, *BSCL2*, and *ZFYVE27*) seem to cause HSP in less than 1% of cases, respectively (8,9). These data guide the molecular

*Correspondence to Dr. Züchner, Department of Human Genetics, John P. Hussman Institute for Human Genetics, University of Miami Miller School of Medicine, Biomedical Research Building, Room 523, LC: M-860, 1501 NW 10th Avenue, Miami, FL 33136. szuchner@med.miami.edu, office: (305) 243-2281, fax: (305) 243-2703.
‡These authors contributed equally to this work.

Conflict of Interest
The authors declare no conflict of interest.

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genetic diagnosis of HSP as expanding the characteristic genotypic spectrum is important for the interpretation of genetic testing results.

Interestingly *ATL1*, *SPAST* and *REEP1* may have directly related functional roles: 1) The atlastin protein has been demonstrated to be a binding partner of spastin (10,11); 2) Atlastin has been implicated in shaping the endoplasmic reticulum (ER) tubular network in concert with REEP1 (12); and 3) A recent study suggests that REEP1, atlastin and spastin interact within the ER and determine ER morphology via interactions with microtubules (13). Thus, beyond being the three most common HSP genes, mutations in *ATL1*, *SPAST* and *REEP1* point to a common biological process disrupted in HSP.

Here we present the results of a mutation screen of 120 HSP patients, in which we identified 20 novel mutations in the two most common HSP genes *SPAST* and *ATL1*. These data include findings from a previous *REEP1* screen in 79 HSP patients (6), which has been expanded by 41 additional cases. This study is unique in that we report the frequency of the three most common HSP genes in a single large cohort. We anticipate that these results will contribute to the molecular genetic understanding of HSP and the genetic spectrum important for clinical diagnosis.

Materials and Methods

We collected a large sample of 120 unrelated HSP cases with a family history of HSP and isolated patients of primarily European descent. Available clinical information on these samples is given in Table 1. Mutations in *REEP1* from 79 of these patients have been previously published (6). Informed consent was obtained from all individuals and the Institutional Review Board (IRB) at the University of Miami Miller School of Medicine approved the study. All individuals were seen by a board certified neurologist. Individuals displaying clinical features attributable to disorders other than HSP were excluded from the study.

Blood (≥24ml) was collected in either EDTA or acid citrate dextrose tubes from participating individuals by venipuncture, and DNA was extracted by the Biorepository of the Hussman Institute for Human Genomics at the University of Miami. Primers flanking each exon (and neighboring intronic sequences) of *SPAST*, *ATL1* and *REEP1* were designed using Primer3 (http://frodo.wi.mit.edu/primer3/) and are available upon request. Exons and flanking intronic sequences were amplified on the Applied Biosystems (ABI, Foster City, CA) Veriti 96-well Fast Thermal Cyclers using a touchdown protocol. PCR purification was completed with QuickStep™2 SOPE resin (Edge BioSystems). Sequencing was performed using ABI BigDye®Dye Terminator Cycle Sequencing Kit on an ABI 3730 sequencer. Sequence traces were analyzed using Sequencher®ver. 4.8 (Gene Codes Corporation). Each nucleotide variation identified was confirmed by completing PCR amplifications and subsequent bi-directional sequencing on fresh aliquots of sample DNA. All mutations were also analyzed with PolyPhen, which is software that predicts the functional significance of amino acid changes on proteins (genetics.bwh.harvard.edu/pph/).

To screen for copy number variations, multiple ligation dependent probe amplification (MLPA) was performed with the Salsa kit P165-B1 HSP (MRC-Holland, Amsterdam) according to the manufacturer's protocol. The kit contains probes for all exons in the *SPAST* and *ATL1* genes. 150ng of sample DNA was analyzed on an ABI 3130XL genetic analyzer (ABI, Foster City, CA) using LIZ 600 as an internal size standard. Fragment analysis was performed using GeneMapper software v4.0 (ABI, Foster City, CA). Results of peak areas were exported to Coffalyser® MLPA data analysis software (MRC-Holland, Amsterdam). In Coffalyser®, the relative peak area (RPA) was calculated and compared with controls.

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This program identifies a peak as normal when showing a 0.7–1.3 ratio with normal controls, as a heterozygous deletion when showing a ratio <0.7, and as a duplication when showing a ratio >1.3. HSP samples with previously published large deletions were used as positive controls.

Results

In a screen of 120 HSP patients, we identified mutations in *ATL1*, *SPAST* and *REEP1* in 44 patients. In our sample, mutations in these three genes account for 36.7% of all HSP cases. None of the identified novel changes were present in 100 control samples or 276 chromosomes studied in the 1000 Genomes Project (dbSNPv131).

Mutations in SPAST

We identified 30 mutations in *SPAST* in 33 HSP patients (Table 2). Of the 30 mutations, 18 were novel while 12 have been previously described (14–19). Two patients (families 25028, 2345) each harbored two separate mutations in *SPAST*; however, we had no DNA available to test whether these changes occur on the same of opposite chromosomes. Five mutations were associated with complicating symptoms, including peripheral neuropathy, ataxia, seizures, and dysarthria (Table 2). Applying MLPA we identified one patient with a large deletion in exon 16, which has been previously described (20).

Mutations in ATL1

Five patients were identified to carry mutations in *ATL1*, representing four distinct missense mutations, Y196C, R239C, V252I and R495W. Two of the four changes are novel, while the R239C and R495W variants have been described previously (21–23). While the V252I mutation is predicted to be benign using PolyPhen, it falls within the GBP/Ras-like GTPase domain in close proximity to the majority of reported *ATL1* mutations. Two of the four mutations were associated with additional symptoms including seizures, ataxia, and MRI hyperintensities (Table 2). MLPA analysis of *ATL1* revealed no copy number variants.

Mutations in REEP1

Of the 120 samples, 79 were previously screened for REEP1 mutations (7). In the previous study we identified six mutations in REEP1, including two variants that fell into the 3'-UTR and that are predicted to affect a binding site for microRNA-140. All mutations were associated with a pure HSP phenotype. We expanded the REEP1 screen to include an additional 41 patients but did not detect any additional changes. We did not test for CNVs in REEP1 because of their previously reported very low frequency (6).

Discussion

Genetic testing strategies in pure HSP are typically guided by the known prevalence of *SPAST*, *ATL1*, and *REEP1* mutations. Our data are consistent with previously reported mutation frequency estimates of *SPAST*, *ATL1* and *REEP1* with mutation frequencies of 28.3%, 4.2%, and 5%, respectively. When considering modes of inheritance in our cohort, *SPAST* mutations account for 30.1% and REEP1 mutations account for 5.8% of autosomal dominant HSP cases, whereas *ATL1* mutations account for only 3.8%. Alternatively, when cases are classified into pure and complicated forms, mutations in *SPAST* and *ATL1* account for 31.8% and 18.2% of complicated cases, respectively. Similar to the reported literature, *SPAST* mutations are associated with a wide range of age of onset, from 1 to 63 years of age with a mean age of 27.6 years, although the age of onset is greater than 30 years in over half of our patients. Mutations in *ATL1* are associated with a younger age of onset, spanning from eight to 36 years, with an average of 20.8 years. While the age of onset of 35 and 36

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years associated with the two novel *ATL1* mutations (families 2315, 25011) are much later than the normally observed childhood onset, others have reported similarly late ages of onset, even with mutations traditionally associated with early onset HSP (24,25). While not typical, this data suggests along with previous reports that additional factors influence the severity and age of onset of HSP associated with mutations in ATL1.

Consistent with previous reports, the majority (19 out of 30) of *SPAST* mutations fall within the AAA domain (Fig. 1), while the remaining changes fall within secondary clusters as described by Shoukier et al. (26). Likewise, the two novel *ATL1* mutations fall within the conserved guanylate-binding protein domain in which most previously described mutations also cluster (21,27).

In our cohort, the allele frequency of two hypothesized modifiers in *SPAST*, S44L and P45Q (3.3% and 0.83%, respectively) is higher than the minor allele frequencies observed in the general population in North America (0.6% and 0.2%, respectively) (15). These findings are consistent with the hypothesis that these alleles are disease associated, possibly modifying severity and age of onset. Interestingly, one of the patients (family 25028) harbors a previously described nonsense mutation (R431X) in addition to a novel P97T mutation (16,28). Like the S44L and P45Q mutations, the P97T mutation resides towards the N-terminus of *SPAST*, away from the conserved domains where most mutations appear to cluster, and it is predicted by PolyPhen to be “benign” (Table 2) (15). The age of onset associated with the P97T and R431X mutations in our study is 18 years compared to 33 years in previous reports (16). Thus, the P97T allele may be a disease modifying allele similar to S44L and P45Q. Additionally the P97T mutation was identified in two affected members of a separate family (25006), suggesting that the heterozygous change may be causative itself. Both individuals had an age of onset below 20 years of age. The modifying role of apparently benign missense mutations in the N-terminus of *SPAST* lends itself as a model for the study of polygenic disease traits, and future genetic screens will be necessary to confirm the role of potentially modifying mutations such as P97T (29–32). The identification of the p.V201D change (family 1676) in exon 4 of SPAST, is inconsistent with an otherwise absence of reported exon 4 mutations. Although phosphorylation and glycosylation sites have been predicted to exist within exon 4 none appear to fall on residue V201 (33). In the absence of available segregation data it is difficult to decide whether the p.V201D change is indeed a mutation or a rare variant not associated with HSP. Likewise, the benign prediction scores for the L314S, L360V and T550I may be incorrect. The T550I change has been previously described to be a causative mutation and changes in residues in close proximity to L360V (P361R and S362C) have also been reported to be causative (28,34,35).

In all we are adding 18 novel *SPAST* and 2 novel *ATL1* mutations to a large number of reported mutations. These results further underscore the impressive allelic heterogeneity of the studied HSP genes. The absence of additional *REEP1* mutations in the 41 patient samples not included in the original *REEP1* screen decreases previous prevalence estimates of 6.5% down to approximately 5.0% (7). Surprisingly, in our cohort *REEP1* accounts for a greater percentage of HSP cases than *ATL1* (5% versus 4.2%). The importance of screening large deletions in *SPAST* was previously reported (20), and accordingly we identified one patient with a large deletion spanning exon 16. While large deletions in *SPAST* were previously estimated to account for approximately 18% of AD HSP (36), our results indicate that these deletions may account for a much smaller percentage of cases (~1%). In conclusion, our observations are largely consistent with previously reported mutation frequencies with respect to mode of inheritance, age of onset and associated clinical findings, and substantiate the diagnostic utility of genetic testing of *SPAST*, *ATL1*, and *REEP1* in HSP.

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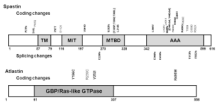

Figure 1.

Schematic of conserved domains of *spastin* (SPAST) and *atlastin* (ATL1) showing that the majority of mutations cluster in the AAA domain and the GBP/Ras-like GTPase domain respectively. Novel mutations are shown in boldface, coding changes are listed above the protein depiction and splicing mutations are listed below. TM = transmembrane domain; MIT = Microtubule Interacting and Trafficking molecule domain; MTBD = Microtubule-Binding Domain; AAA = ATPases Associated with a wide variety of Activities.

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Table 1

Sample Characteristics

Gender	**Patients**	**Percent**
Male	66	55%
Female	54	45%
Ethnicity		
European	118	98.3%
Non-European	2	1.7%
Age of Onset		
Range in years:	1–63	
Average in years:	26.4	
Clinical Phenotype		
Pure	98	81.7%
Complicated	22	18.3%
Inheritance Pattern		
Autosomal Dominant	103	85.8%
Autosomal Recessive	8	6.7%
Sporadic	9	7.5%
Patients with mutations	44	36.7%
Spastin (SPG4)	33	27.5%
Atlastin (SPG3A)	5	4.2%
REEP1 (SPG31)	6	5.0%

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Table 2

Summary of mutations identified in *spastin* (SPG4), *atlastin* (SPG3A) and *REEP1* (SPG31) in our sample.

Family	Exon (Intron)	Mutation Type	Mutation	Predicted Protein Change	Consequence (PolyPhen/ Splice Prediction1)	PolyPhen[1]/ Splice[2] Prediction Score	Inheritance Pattern	Age of Onset	Pure/ Complicated	Reference/ Novel
SPASTIN (SPG4)										
25026	1	Truncating	c.81insC	p.P27fs	Frameshift		AD	50	Complicated (ataxia)	Novel
1735, 2151, 25010, 25033	1	Missense	c.131C>T	p.S44L	Benign	0	AD, AD, AD, AD	10, 63, 6, 9,	Complicated (neuropathy): 1; Pure: 3	Lindsey et al. (2000)
2526	1	Missense	c.134C>A	p.P45Q	Benign	0	AD	40	Complicated (dysarthria)	Svenson et al. (2004)
25006, 25028‡	1	Missense	c.289C>A	p.P97T	Benign	0.161	AD	13,18	Pure	Novel
25003	1	Truncating	c.334G>T	p.E112X	Truncation		AD	55	Pure	Patrono et al. (2005)
1676	4	Missense	c.602T>A	p.V201D	Benign	0.45	AD	23	Pure	Novel
25017	−4	Truncating	c.683-1G>T	p.E228fs	AS prediction change	0.90>0.00	AD	1	Pure	Novel
2184	5	Truncating	c.843_846dupATCT	p.S282fs	Frameshift		AD	21	Pure	Novel
1983	6	Missense	c.913_923del/insCCAATTGCACT	p.[T305P, T306I, T308L]	Probably damaging	1.658	AD	25	Pure	Novel
1831	6	Missense	c.941T>C	p.L314S	Benign	0.349	AD	NA	Pure	Novel
2345*	7	Missense	c.1078C>G	p.L360V	Benign	1.476	AD	40	Pure	Novel
2345*	−7	Truncating	c.1098+1G>C	p.E366fs	DS prediction change	1.00>0.00	AD	40	Pure	Novel
1974	−9	Truncating	c.1245+1G>A	p.Y415fs	DS prediction change	0.98>0.00	AD	49	Pure	Novel
1838	−9	Truncating	c.1245+4A>G	p.Y415fs	DS prediction change	0.98>0.82	AD	45	Pure	Novel
25044	−10	Truncating	c.1322-31del29bp	p.I440fs	AS prediction change	0.76>0.00	AD	25	Pure	Novel
25045	11	Missense	c.1276C>G	p.L426V	Possibly damaging	1.617	AD	5	Pure	Svenson et al. (2004)
25028‡	11	Truncating	c.1291C>T	p.R431X	Truncation		AD	18	Pure	Fonknechten et al., (2000)

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Family	Exon (Intron)	Mutation Type	Mutation	Predicted Protein Change	Consequence (PolyPhen/ Splice Prediction1)	PolyPhen[1]/ Splice[2] Prediction Score	Inheritance Pattern	Age of Onset	Pure/ Complicated	Reference/ Novel
2450	11	Truncating	c.1323_1328delAGTTGA	p.[E441del; V442del]	Probably damaging	2.674	AD	18	Pure	Novel
2104, 25053	11	Missense	c.1378C>T	p.R460C	Probably damaging	2.97	AD	30, 35	Pure	Falco et al. (2004),
2376	11	Missense	c.1391A>C	p.E464A	Probably damaging	2.343	AD	1.5	Complicated (epilepsy)	Novel
25009	11	Truncating	c.1403G>T	p.G468X	Truncation		AD	5	Pure	Novel
2417	12	Missense	c.1492T>G	p.R498G	Probably damaging	2.745	AD	25	Pure	Novel
2769	13	Missense	c.1495C>T	p.R499C	Probably damaging	2.97	AD	51	Pure	Hazan et al. (1999)
25024	13, (13)	Truncating	c.[1535delAG, 1535+1delG]	p.E511fs	Frameshift		AD	32	Pure	Novel
25012	−14	Truncating	c.1538-1G>T	p.T513fs	Splicing	0.99>0.00	AD	14	Complicated (peripheral neuropathy)	Novel
25032	15	Missense	c.1649C>T	T550I	Benign	1.01	AD	33	Pure	Orlacchio et al. (2008)
25031	15	Missense	c.1673T>G	p.L588R	Possibly damaging	1.87	AD	32	Pure	Novel
25029	15	Missense	c.1676G>A	p.G559D	Possibly damaging	1.611	AD	41	Pure	Zhao et al. (2001)
25039	15	Truncating	c.1684C>T	p.R562X	Truncation		AD	30	Pure	Sauter et al. (2002)
25020	16	Truncating	c.1688-?_17281?del	p.562fs	Frameshift		AD	35	Pure	Beetz et al. (2004)
ATLASTIN (SPG3A)										
2315	6	Missense	c.587A>G	p.Y196C	Probably damaging	2.995	AD	35	Complicated (MRI white matter changes, seizures)	Novel
1839, 2221	7	Missense	c.715C>T	p.R239C	Probably damaging	2.514	AD	10,15	Pure	Zhao et al. (2001)
25011	8	Missense	c.757G>A	p.V252I	Benign	0.116	AD	36	Complicated (ataxia)	Novel
1986	12	Missense	c.1483C>T	p.R495W	Probably damaging	2.695	AD	8	Pure	Scarano et al. (2005)
REEP1 (SPG31)										

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Family	Exon (Intron)	Mutation Type	Mutation	Predicted Protein Change	Consequence (PolyPhen/ Splice Prediction1)	PolyPhen[1]/ Splice[2] Prediction Score	Inheritance Pattern	Age of Onset	Pure/ Complicated	Reference/ Novel
2189	2	Missense	c.59C>A	p.A20E	Probably damaging	1.747	AD	3–62	Pure	Züchner et al. (2006)
2036	4	Truncating	c.182-2A>G	p.W61fs	Frameshift		AD	5–18	Pure	Züchner et al. (2006)
2299	6	Truncating	c.507delC	p.P170fs	Frameshift		AD	3–60	Pure	Züchner et al. (2006)
2369	6	Truncating	c.526delG	p.G176fs	Frameshift		AD	18	Pure	Züchner et al. (2006)
2354	3’-UTR	--	c.606+43G>T	miR-140 target site change	microRNA binding change		AD	6	Pure	Züchner et al. (2006)
1959	3’-UTR	--	c.606+50G>A	miR-140 target site change	microRNA binding change		AD	40	Pure	Züchner et al. (2006)

‡,* Symbols denote samples with multiple distinct mutations in the same gene.

[1] PolyPhen: predicts the functional effect of non-synonymous mutations ranking changes <=0.5 as benign, >0.5 and <2.0 as possibly or probably damaging depending on residue chemistry, and >=2.0 as probably damaging.

[2] Predictions from splice site analysis from NNSPLICE ver. 0.9 (at http://www.fruitfly.org/seq_tools/splice.html) ranging from 0 (no site predicted) to 1.0 (splice site likely). The first number represents splice site prediction without variation, the number after the > symbol represents the splice site prediction with the variant.

DS = donor site; AS = acceptor site; fs = frameshift; miR = microRNA; AD = autosomal dominant; AR = autosomal recessive; NA = not available