ORIGINAL CONTRIBUTION

Spectrum of *SPG4* Mutations in a Large Collection of North American Families With Hereditary Spastic Paraplegia

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Background: Hereditary spastic paraplegia (HSP) is a neurodegenerative disease characterized by progressive spasticity and weakness of the lower limbs. The most common form of HSP is caused by mutations in the *SPG4* gene, which codes for spastin, an adenosine triphosphatase with various cellular activities (AAA) protein family member.

Objective: To investigate a large collection of predominantly North American patients with HSP for mutations in the spastin encoding gene, *SPG4*.

Methods: DNA from 76 unrelated affected individuals was studied for mutations by single-stranded conformational polymorphism analysis and direct sequencing. Each new variant identified was then analyzed in 80 control subjects to determine whether the variant is a common polymorphism or a rare mutation. All DNA samples were amplified by polymerase chain reaction, followed by electrophoresis and autoradiography.

Results: We identified 8 novel mutations and 5 previously reported mutations in 15 affected individuals. The novel mutations are 4 missense, 1 nonsense, 1 frameshift, and 2 splice mutations. Two polymorphisms (one in an affected individual) were also identified.

Conclusions: Our collection of families with HSP is different on a genetic level from those previously described. The percentage of our families with a *SPG4* mutation is 10% lower than the 40% estimate of families with autosomal dominant HSP noted to be linked to this locus, and splice mutations are not predominant in our collection. Interestingly, we also identified 2 recurring mutations in specific populations (R562Q and G559D), which may facilitate the development of future spastin diagnostic testing in these populations.

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HEREDITARY SPASTIC paraplegia (HSP), also known as familial spastic paraplegia (FSP) or Strümpell-Lorrain syndrome, comprises a heterogeneous group of neurodegenerative disorders characterized by progressive lower limb spasticity and weakness associated with bladder disturbance in approximately 50% of the cases.[1,2] Clinically, there are 2 types of HSP: the pure form, which is characterized exclusively by leg spasticity often with bladder disturbance; and the complicated form, which includes additional neurological abnormalities such as optic neuropathy, dementia, ataxia, deafness, mental retardation, and extrapyramidal disturbance.[1,2] The main neuropathological feature is axonal degeneration of the distal ends of the longest ascending and descending tracts. This neurodegeneration results in spasticity of the lower limbs, which causes difficulty walking; in severe cases, the patients become wheelchair bound.[3,4] The age of onset varies greatly, even within families, from early childhood to the mid-80s. The genetic heterogeneity in individuals with HSP is demonstrated by the number of loci (*SPG1* through *SPG17*) that have been mapped. These include 3 X-linked,[5-7] 9 autosomal dominant,[8-16] and 5 autosomal recessive[17-21] loci. Of all described families, 40% are linked to an autosomal dominant locus known as *SPG4* (MIM182601).[22] The defective gene for *SPG4* was cloned recently and encodes a 616–amino acid protein named spastin. The protein is an adenosine triphosphatase associated with various cellular activities (AAA) protein family member with a characteristic AAA cassette in the C terminus from amino acid 342 to amino acid 599. According to computer predictions, the protein possesses a nuclear localization signal, Walker motif A and B, an AAA minimal consensus sequence, leucine zipper motifs, and a helix-loop-helix domain. Furthermore, the protein shows strong homology to 2 yeast proteins (Yta6p and Sap1) and to the mouse Skd1 protein. Recent evidence[23] suggests that spastin might interact with tubulin, but

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PATIENTS AND METHODS

PATIENTS

The participants included 5 control subjects and 76 unrelated patients from families with HSP who were available in our laboratory. The families FSP4 (Z_{max}=1.09 [G. A. Rouleau, PhD, MD, unpublished data, 2000]), FSP7 (Z_{max}=2.7), and FSP28 (Z_{max}=0.77)[33] had previously shown suggestive linkage to the *SPG4* locus. (Z_{max} signifies the maximum logarithm of odds score in genetic linkage analysis.) The families were diagnosed as having either autosomal dominant HSP (ADHSP) (49 families) or autosomal recessive HSP (11 families), and the other 16 families are small families in which the pattern of inheritance is unclear. All probands were seen by a neurologist, and the diagnostic criteria were lower limb spasticity in the absence of any evidence for a structural lesion or demyelination. Our collection of patients consists of mostly French Canadian and other white (Canadian and US) families, with the exception of 2 Middle Eastern families. Patients and controls gave informed and written consent. Genomic DNA from these individuals was prepared from peripheral blood leukocytes by standard extraction methods.

MUTATION SCREENING

Single-Stranded Conformational Polymorphism Analysis

Polymerase chain reaction (PCR) primer pairs were designed from the genomic DNA sequence of the human *SPG4* gene (GenBank accession numbers AL121655 and AL121658), as shown in **Table 1**.

The 17 coding exons were amplified by PCR using approximately 20 ng of genomic DNA and radiolabeled deoxyadenosine triphosphate in a total volume of 13 µL for single-stranded conformational polymorphism (SSCP) analysis. The reactions were performed in thermocyclers (PerkinElmer, Inc, Forest City, Calif), starting with an initial denaturation of 5 minutes at 94°C followed by 30 cycles of 30 seconds' denaturation, 30 seconds' annealing at the primer-specific temperature, and 45 seconds' extension at 72°C. Electrophoresis of PCR products was performed on 9.5% polyacrylamide (5% glycerol) and on 50% mutation detection enhancement gels (Biowhittaker Molecular Applications, Rockland, Me), followed by autoradiography. Samples showing altered migration patterns were selected for further analysis.

Sequencing

Genomic DNA from selected samples was amplified in 50 µL of nonradiolabeled PCR mix. The PCR products were purified with a gel extraction kit (QIAEX11; QIAGEN, Mississauga, Ontario), after which the purified fragments were sequenced using a thermosequenase phosphorus 33 cycle-sequencing kit (United States Biochemical Corp, Cleveland, Ohio) and run on 6% polyacrylamide denaturing gels.

Variations identified by sequencing were analyzed for cosegregation in family members when available. All newly identified disease-associated variations were examined for presence in 80 white controls (160 chromosomes) by SSCP analysis.

its main function remains unknown despite its homology to other proteins and functional domains. To date, more than 50 mutations have been reported in the spastin encoding gene, including splice-site, nonsense, missense, and frameshift mutations (insertion and deletion), affecting either directly or indirectly the conserved AAA cassette,[24-32] suggesting the functional importance of the AAA cassette. No clear genotype-phenotype correlation has been reported, but most articles[23] suggest that *SPG4* is caused by the haplo-insufficiency of spastin, which implies a critical spastin threshold.

Herein, we report 8 novel mutations and 2 novel polymorphisms identified in the spastin encoding gene and the recurrence of 2 mutations in 2 populations, which could be important for the development of large-scale *SPG4* mutation detection efforts.

RESULTS

One affected member from each of the 76 kindreds was screened for *SPG4* mutations. Most families were too small to test for linkage, and 3 families had shown suggestive linkage to the *SPG4* locus in a previous study.[33] We identified 13 mutations and 2 polymorphisms in 16 families (**Table 2**). Our results show 8 novel mutations in the *SPG4* gene: 1 frameshift (687delG), 2 splicing (A1538-2G and G1854-1C), 4 missense (C1321T, C1591T, G1810A, and G1801A), and 1 nonsense (G1425T) mutation (**Table 3**). Five previously reported mutations were also found in our collection. The transmission of these mutations correlates with the clinical status of the families, except for some carrier individuals who are asymptomatic, which could be due to age-dependent penetrance, incomplete penetrance, or simply subtle symptoms. Interestingly, mutations were identified in only 2 of 3 families previously linked to *SPG4* (families FSP4 and FSP7). Seven of the novel mutations are in the AAA cassette spanning exon 7 to 16.[22] The last novel mutation is a 1–base pair deletion in exon 3, which results in a frameshift leading to a premature stop codon, truncating the protein before the AAA cassette. This mutation was found in individual R11540 and is one of the rare mutations occurring outside of the AAA cassette of spastin,[29] and further suggests the importance of the AAA cassette in spastin function. DNA for the rest of the family was unavailable for testing of cosegregation. The other nonsense mutation, found in individual R12447, creates a stop codon in the third conserved domain of the protein, which results in a protein lacking the predicted helix-loop-helix domain and a leucine zipper motif.

This study identified 4 novel missense mutations, all occurring in the AAA cassette. The mutation C1321T in exon 9 changes a serine (a polar and hydrophilic amino acid) into a leucine (a hydrophobic amino acid). A mutation in exon 12 leads to a C1591T change, where proline with a cyclic ring is substituted by a hydrophobic linear amino acid, which occurs close to the conserved

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Table 1. Primers Designed for Amplification of All 17 Exons

Exon	Forward Primer	Reverse Primer	Size, bp*
1A	CCACCGACTGCAGGAGGAGA	GCGCCGCCGGAGCCTTTCTTCTTC	260
1B	TTATGGCGGCGGCGGCAGTGAGAG	CCATGAGGGCGCGGGAGAAGC	292
1C	CACCTGGGGCTCCTCTTC	GACCCCACCGCCTTCTT	263
2†	TTTTTATGTATTACCTCTCAA	AAAAATAAATAAATAAATAAATAG	266
3	CTCCCCATGAAAGTAGTT	ATGTTAAAAAGCCTGGAC	290
4	TATCATGTAACAATCTGGTA	TTATAAAATCAAATCAACAT	307
5	TTTTCTAATCACAATGGT	TATGATCAACTTAAGCAGGAAT	309
6	ATGTTAGGTTGTATTTTCA	GTATTTATTATCTATTTCACTCCT	269
7	TGTCATAGGGCTTAGGCTTCA	ATGGATTCAGTAACAGATGGTATT	226
8	CTGTTTGGGAAGATGCT	GTAAATAATAGACTCAAGGACAAG	273
9†	GCATGAGCCACCACACCTG	AGATAAGCTCCTAGAAAAATA	316
10	GTGCTAGATTTTCAACATA	GCCCTTCTTTAAAACTTCTTCC	270
11	ACTCACATAGCTTGGTCTT	AGGAAAATATACTAAAATG	196
12	ATGGCCAAGGTTAAAAATACAA	CTGGAAGAAAATAGTGAAT	281
13	CTTTTCCTGTCATTTGCTGTTTC	TTAATATTGTCAAGATGGTAGTTC	186
14	TCGGGAGGCTGAGATGG	AATAAATAAAAGCTGTAAGATAAA	300
15	AAAAAGCGGGAGGGGAAATA	TGGGCAACAGACTGAGACC	248
16†	ATTGTACTTGGTTTTGCCCTTCA	GAGCCGATATCATGCCAGACT	259
17	ACCACCATATACCTGTTGAT	CTGTTTCTGTAGCCGATGAC	263

*bp indicates base pair.
†These exons have also been amplified using primers by Lindsey et al.[27]

Table 2. Clinical Information of the Probands*

Proband	Age of Onset, y†	Gait	Upper Limb Hyperreflexia	Bladder Disturbance	Babinski Reflex	Additional Comments
Fam33	. . .	No mobility problems	+	−	+	. . .
R3428	Childhood	Unable to walk	−	−	−	Footdrop
R4650	50s	Problems running	−	+	+	Fecal incontinence, nocturnal myoclonus, and restless legs
R4888	40s	Unable to walk	−	−	−	. . .
R5913	Childhood	Unable to stand	−	+	. . .	Fecal incontinence
R7050	. . .	No mobility problems	+	−	+	Patient unaware of symptoms
R9109	40s	Problems walking	+	−	+	Dysphagia
R9259	25	Able to run	−	+	+	. . .
R11540	37	Problems running	+	−	+	Arthritis
R11872	Childhood	Uses a walker	−	−	. . .	Late-onset atypical seizures and memory loss
R12320	70	Problems walking	−	+	+	. . .
R12416	60	Unable to walk	+	−	−	. . .
R12447	40	Trips often, but can still walk	+	+	+	Assymetrical spasticity, depression, dysarthria, nystagmus, and footdrop
R13912	Childhood	Problems walking	−	−	. . .	Dysarthria

*Relevant clinical data for individual 78 are unavailable. All probands listed experienced lower limb hyperreflexia. + indicates present; −, absent; and ellipses, data not available.
†As observed by the patient.

helix-loop-helix domain. The mutation G1801A (in exon 15) gives rise to an amino acid change from glutamic acid to the smaller aspartic acid in the conserved 6226 domain. This mutation was found in 2 French Canadian families with HSP. Last, a missense mutation, G1810A, in exon 15 was found in 2 white US families, and changes a basic amino acid (arginine) to an uncharged amino acid (glutamine). The novel missense mutations all occur in a domain or near a domain of the AAA cassette, suggesting the importance of conserved amino acid structure in this region. None of the variants were found in 160 control chromosomes by SSCP analysis. There is only one report[31] of a coding polymorphism, and it is not the same as any of the previously mentioned mutations.

We found 2 new splice-site mutations (A1538-2G and G1854-1C) in the acceptor splice site of exons 11 and 15, respectively.[34] For both mutations, the predicted truncated protein lacks a part of the AAA cassette, which probably renders the protein nonfunctional and causes disease. Another possibility is that the splicing machinery uses an alternative cryptic splice acceptor, which results in a defective protein.

We also detected 2 possible polymorphisms (G1417A and G1619+16T). One polymorphism leads to an amino

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Table 3. Summary of Mutations Identified in Probands With Hereditary Spastic Paraplegia

Proband	Location*	Mutation†	Amino Acid Change‡	Consequence	Origin
R11540	Exon 3	687delG	PTC + 7 aa	Frameshift	French Canadian
R4888	Exon 6	G1004A[31]	P293P	Possible mutation of splice enhancer or silencer?	White (United States)
R11872	Intron 8	G1298 + 1A[27]	. . .	Splicing mutation	White (United States)
R7050	Exon 9	A1367G[31]	K414K	Splicing mutation	French Canadian
R12416	Exon 9	C1321T	S399L	Missense	French Canadian
R5913	Exon 10	C1401G[24]	L426V	Missense	White (United States)
R12447	Exon 10	G1425T	Q434stop	Nonsense	French Canadian
R13912	Intron 11	A1538-2G	. . .	Splicing mutation	French Canadian
R9109	Exon 12	C1591T	P489L	Missense	French Canadian
R9259	Exon 15	G1801A	G559D	Missense	French Canadian
R12320	Exon 15	G1801A	G559D	Missense	French Canadian
Fam33	Exon 15	C1809T[24]	R562stop	Nonsense	White (United States)
78	Exon 15	G1810A	R562Q	Missense	White (United States)
R4650	Exon 15	G1810A	R562Q	Missense	White (United States)
R3428	Intron 16	G1854-1C	. . .	Splicing mutation	White (United States)

*The nucleotide location was assigned using *SPG4* complementary DNA (information available at: http://www.genoscope.cns.fr. Accessed November 1999).
†Reference numbers indicate previously reported mutations.
‡In 3 cases, no amino acid (aa) changes are listed because in splicing mutations, the entire protein will be of a different size and it cannot be predicted what the protein will consist of.

acid change (R431Q) in the third conserved domain of spastin of a spouse of an affected individual included in the screening as a control. This individual was seen by a neurologist and was not affected with HSP. The proband of this family is individual R12320, who has a missense mutation (G1801A). The other polymorphism is an intronic substitution 16 nucleotides away from the exon in an affected individual and does not show cosegregation in the family.

COMMENT

Fifteen mutations, 8 that are novel, were identified in the *SPG4* gene of patients from a collection of 76 predominantly North American families with HSP. Interestingly, mutations in the spastin encoding gene were found in only 15 (31%) of the 49 known families with ADHSP who were analyzed, which suggests that our collection of families represents a subpopulation that does not exactly conform to the 40% estimate of families with ADHSP linked to *SPG4*. A previous report[33] of linkage analysis from our laboratory showed that only 14% of the families in that study[33] linked to *SPG4*, which correlates with the lower percentage of mutations found in the screening. Some of the 16 small families with an unclear pattern of inheritance could also be families with ADHSP, which would decrease the percentage of our families linked to *SPG4*. Furthermore, 11 families with recessive disease were included in the screening because Lindsey and colleagues[27] reported a homozygous mutation in spastin, S44L, in a kindred with autosomal recessive HSP. There were no mutations detected in any of our families with recessive disease. These data suggest that *SPG4* mutations may be responsible for a smaller fraction of ADHSP than previously reported, and that spastin gene screening is only worthwhile in kindreds showing clear autosomal dominant inheritance. However, it is possible that our screening underestimated the fraction of *SPG4* mutation–positive families because SSCP analysis is only 80% to 100% effective in detecting mutations,[35] and mutations occurring in the noncoding regions, such as the promotor and other regulatory sequences, would not be detected by our screening, which analyzed the exons and the intron-exon junctions. Nevertheless, our methods are similar to those used in other *SPG4* mutation screenings.

While most reports of *SPG4* mutations show a predominance of splice mutations, missense mutations were the most frequent ones found in our study. Only 3 splice mutations were found of 13 mutations identified in affected individuals. This could be related to the fact that our families are North American, and, to our knowledge, no large-scale study has ever been performed on this population. We do not think it is likely that variants in the splice junction were not detected because the primers used in this study included approximately 30 base pairs of the flanking intronic regions. The missense mutations found in affected individuals were not found in 160 control chromosomes, and showed cosegregation with the disease phenotype in the respective families. All affected individuals in a *SPG4* mutation–positive family had the identified mutation. Because HSP has incomplete and age-dependent penetrance, some seemingly unaffected individuals (Fam33, R7050, and some family members tested for cosegregation) in our screening carried a mutation in the spastin encoding gene. In addition, it is estimated that 25% of individuals are asymptomatic or unaware of their symptoms.[36] These data strongly support a causative role for these mutations in individuals with HSP.

The spastin gene is highly conserved through species, which suggests that amino acid changes, especially when present in the AAA cassette, are likely to have a consequence on protein function. There are no reports of a polymorphism in the coding region in this gene except for one variant, G1004A,[31] which was first detected in a proband of a family with HSP but not in 80 controls. Our study identified the same variant in a second affected individual. Unfortunately, no other affected individuals in this kindred are available for testing, but the variant was not present in 80 controls. Because this vari-

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ant has not been detected in 320 control chromosomes and has twice been identified in affected individuals, it is likely that the variant is a mutation involved in disease etiology. The variant, which does not change the amino acid sequence, could possibly disrupt an exonic splicing enhancer or silencer, thereby affecting the patterns or efficiency of messenger RNA (mRNA) splicing.[37] Future mRNA work is required to further investigate the effects of this mutation. Two polymorphisms, one of which leads to an amino acid substitution, were also identified in this study. The characterization of variants as polymorphisms should be confirmed by mRNA reverse transcriptase–PCR analysis and studies of larger control populations.

Five mutations were identified in individuals with complicated HSP showing atypical symptoms, such as restless legs, atypical seizures, and dysarthria. One interesting individual (aged 45 years) has a nonsense mutation in exon 10, with severe manifestation of disease showing spasticity, footdrop, and signs of ataxia, dysarthria, and nystagmus. The severe phenotype could be classified as complicated HSP or spastic ataxia and suggests that this gene might also be involved in other clinical entities. Contrary to the nonsense mutation previously mentioned, a conservative mutation in exon 10 for patient R5913 (aged 66 years) caused early-onset progressive spasticity, rendering the patient bedridden, with rather uncommon fecal incontinence (Table 2). Last, there are 2 patients (R7050 and Fam33) who have no mobility problems and were unaware that they are affected. They have a previously reported splice mutation[31] and a nonsense mutation, respectively. One would expect that the individual with the nonsense mutation in exon 15 and, therefore, lacking the C-terminal end of the AAA cassette, might have a relatively severe phenotype, but remarkably that is not the case. The fact that a splice alteration in acceptor exon 15 causes disease suggests that the C-terminal part of the AAA cassette is important for function and that having even a part of the cassette is not sufficient for normal function. More in-depth phenotype-genotype correlation studies would be necessary to further support that there is no actual correlation.

In addition, patients R9259 and R12320 have the same mutation but different phenotypes, particularly age of onset. The clinical variability that occurs in individuals with the same or similar mutations might be due to modifier genes; this hypothesis is supported by a report[38] of mutations found in the *EAAT2* gene (already involved in amyotrophic lateral sclerosis) in some patients with HSP. The modifier gene theory can also partly explain why there are reports of complicated HSP and pure HSP at the *SPG4* locus. The hypothetical modifier might be related to the conserved AAA cassette. Spastin bound to adenosine triphosphate could bind a modifier or activate a secondary gene, which allows for greater expressivity of the phenotype. Unfortunately, the function of spastin can only be hypothesized.

To our knowledge, this study is the first to identify 2 recurring mutations. The G1801A mutation was identified in 2 seemingly unrelated French Canadian families, and the G1810A mutation was found in 2 seemingly unrelated white US families. This suggests that these 2 mutations show a higher frequency in the specific populations, which could be helpful for future *SPG4* diagnostic testing in these populations. It is important to identify frequent mutations in specific populations because the spectrum for spastin gene mutations is so great, making efficient genetic testing only feasible in subpopulations. There were 24 other French Canadian families included in the screening who did not show any significant variation in the *SPG4* gene. These families are not all from the same region in Quebec, but are assumed to belong to the general French Canadian population. The results suggest that there is at least one more defective gene responsible for HSP in the French Canadian population.

In conclusion, this report presents 8 novel mutations in the *SPG4* gene, contributing to the large spectrum of mutations found in this gene. Continuing to identify new mutations is important, particularly for developing diagnostic testing programs based on the frequency of mutations in specific populations. Further phenotype-genotype correlations studies are required to unveil the reason behind the great variable expressivity for the *SPG4* form. A better understanding of the function of spastin is vital to discovering a treatment for HSP, and future phenotype-genotype studies and localization studies for the protein will be necessary.

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