Eight Novel Mutations in SPG4 in a Large Sample of Patients With Hereditary Spastic Paraplegia

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Background: Hereditary spastic paraplegia (HSP) is a group of genetically heterogeneous disorders characterized by progressive spasticity of the lower limbs. Mutations in the SPG4 gene, which encodes spastin protein, are responsible for up to 45% of autosomal dominant cases.

Objective: To search for disease-causing mutations in a large series of Italian patients with HSP.

Design: Samples of DNA were analyzed by direct sequencing of all exons in SPG4. Samples from a subset of patients were also analyzed by direct sequencing of all exons in SPG3A, SPG6, SPG10, and SPG13.

Setting: Molecular testing facility in Italy.

Patients: Sixty unrelated Italian patients with pure (n=50) and complicated (n=10) HSP.

Main Outcome Measures: Mutations in SPG4, SPG3A, SPG6, SPG10, and SPG13.

Results: We identified 12 different mutations, 8 of which were novel, in 13 patients. No mutations of any of the other HSP genes tested were found in 15 patients with sporadic pure HSP who did not have mutations in the SPG4 gene.

Conclusions: The overall rate of mutation in the SPG4 gene within our sample was 22%, rising to 26% when only patients with pure HSP were considered. The negative result obtained in 15 patients without mutations in SPG4 in whom 4 other genes were analyzed (SPG3A, SPG6, SPG10, and SPG13) indicate that these genes are not frequently mutated in sporadic pure HSP.

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HEREDITARY SPASTIC paraplegia (HSP) represents a group of inherited disorders characterized by slowly progressive lower limb spasticity, hyperreflexia, and mild weakness.1 The occurrence of additional features allows the differentiation between pure and complicated forms of the disease. Hereditary spastic paraplegia may be inherited as an autosomal dominant, autosomal recessive, or X-linked form. Autosomal dominant transmission is observed in approximately 70% to 80% of families with HSP, among the 10 autosomal dominant HSP loci, SPG4, which encodes spastin protein, accounts for the largest share (18%-45%).2,7 Other genes are now known to be responsible for pure forms of the disease, namely SPG3A,8 SPG6,9 SPG10,10 and SPG13.11 The spastin protein belongs to the AAA ATPase (adenosinetriphosphatase) protein family, which has various cellular activities.2 Like all members of this protein family, spastin carries a typical AAA cassette in the C-terminal domain from amino acid 342 to amino acid 599. The N-terminal region contains a proline-rich region followed by an MIT (microtubule-interacting and trafficking molecules) domain common to proteins involved in endocytosis and trafficking.10,32 Emerging evidence suggests a role for spastin in microtubule dynamics,15-18 but the mechanism by which spastin abnormalities lead to axonal degeneration remains largely unknown. More than 150 different mutations have been identified in SPG4, but the genotype-phenotype correlation is still unclear. Herein we report the results of a mutation analysis in the SPG4 gene that was carried out in a group of Italian patients.

METHODS

PATIENTS

Sixty unrelated Italian patients with HSP (35 sporadic and 5 familial cases) were referred to our testing facility for molecular analysis. Clinical evaluation by the referring neurologists was based on the Harding criterion19 for the definition of clinical status. Disability was assessed on a 5-point scale in which 1 indicates normal or very slight stiffness in the legs and 5, wheelchair bound.7
Haplotype analysis with markers from the SPG4 genomic region was carried out only in families HSP1 and HSP4, and evidence of a common haplotype shared by all affected individuals from each family was obtained. After informed consent was given, blood specimens were obtained from the probands and unaffected subjects. This study was approved by the ethical committee of IRCCS Eugenio Medea of Bosisio Parini, Lecco, Italy.

HAPLOTYPE AND MUTATION ANALYSES

Genomic DNA from peripheral blood leukocytes was obtained with a nucleic acid extraction kit (IsoQuick; ORCA Research, Inc, Bothell, Wash). Haplotype analysis of the family members of kindreds HSP1 through HSP4 was performed using 3 microsatellite markers (D2S400, D2S2351, and D2S367) spanning the SPG4 region. All coding exons of SPG4, SPG3A, SPG6, SPG10, and SPG13 were amplified by polymerase chain reaction with primers designed by us and using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, Calif) according to the manufacturer’s instructions. Sequencing was performed with a kit (Big Dye Terminator; Applied Biosystems) and run on a genetic analyzer (ABI 3100 Avant, Applied Biosystems). Primers for duplication in exon 1 of the SPG4 gene (c.80-98dup19) were as follows: forward primer: 5’-GGACGAGGGAAGAAGAAAGG-3’; and reverse primer: 5’-GCGGGTAGGAGAAATAGTACAGG-3’. The size of the amplified fragments was 160 base pairs (bp) (control) or 179 bp (duplicated). The SPG4 mutation nomenclature is based on National Center for Biotechnology Information Reference Sequence No. NM_014946.3, according to the recommendations of the Human Genome Variation Society. All nucleotide changes reported were checked in a panel of 250 healthy subjects (unless otherwise stated) recruited from the same Italian regions as those of the patients.

RESULTS

We examined 60 unrelated Italian patients, 50 (5 familial and 45 sporadic cases) with pure HSP and 10 sporadic cases with complicated forms of the disease. The patients presented with hyperreflexia and spasticity of the lower limbs, scissors gait, ankle clonus, Babinski sign, and talipes cavus. Disease onset ranged from 20 months to 62 years of age. Other than the typical signs of the HSP phenotype, the 10 patients with complicated forms of the disease showed variable degrees of cognitive deficit (5 patients), cerebellar signs or signs of cerebellar atrophy on cerebral imaging (3), and signs of peripheral neuropathy on electromyography/electroneuronography (2). In the present study, all of the SPG4 mutations were found only in patients with pure HSP disease. Among the sporadic cases, only the parents of patient 5 were available for analysis, but they were negative for the SPG4 mutation. The clinical features of the families and sporadic cases with SPG4 mutations are summarized in Table 1. Brain
Figure 1. Pedigrees and molecular analyses of 3 families with hereditary spastic paraplegia (HSP): HSP1 (A), HSP2 (B), and HSP3 (C). Arrows on the pedigrees and on the electropherograms indicate the proband and mutation site, respectively. The mutations at the DNA and protein levels are listed for each family. In family HSP1 (A), we also conducted gel electrophoresis of the polymerase chain reaction (PCR) products obtained with primers flanking the duplication (dup) in all family members. The band doublet (arrow) represents the heterozygous duplication identified. B indicates the PCR blank; bp, base pair; del, deletion; fs, frame shift; ins, insertion; MW, molecular weight marker V; and NA, that a blood sample was not available.
and spinal cord magnetic resonance imaging results were normal in all patients.

The pedigrees of the families are represented in Figure 1 and Figure 2. In family HSP5 (Figure 2B), only individuals II:1 (the proband) and I:1 were examined clinically; the status of the other family members were reported by the proband. A blood sample was obtained from the proband only.

**MUTATION ANALYSIS**

The sequencing of all exons of the SPG4 gene in all patients allowed us to identify 12 heterozygous mutations, found in 13 patients (5 familial and 8 sporadic cases) (Table 2, and Figures 1 and 2). None of the mutations were found in the 500 control chromosomes. Eight of these mutations were novel and 4 have already been reported (p.I406V, p.R499H, p.R503W, and p.R562X). Mutation p.I406V was detected in all affected individuals of family HSP4 (Figure 2A). The 2 sisters (III:1 and III:2), who had a more severe phenotype than their father (II:2) and an earlier age at onset (2 vs 30 years), carried the modifier variant p.S44L in addition to p.I406V. Haplotype reconstruction and segregation analysis demonstrated that the mutation arose de novo in individual II:2 (data not shown). When we checked the control chromosome panel, the frequency of the p.S44L variant within the Italian population was 0.6% (3/500).

All of the known mutations (p.I406V, p.R499H, p.R503W, and p.R562X) and 3 of the novel ones (p.D493G, p.E533X, and c.1616-2 A/H11022 G [at intron 14]) fall within the AAA domain of the protein (Table 2). The missense mutation p.D493G segregated in all affected members of family HSP3 (Figure 1C). Mutation p.E533X and the splice-site mutation c.1616-2 A/H11022 G (at intron 14) were detected in patients 7528 and 3, respectively, of the sporadic cases (Table 2). The functional consequence of the splice-site mutation could not be determined owing to the lack of suitable biological material. However, the splice-site mutation is likely to produce a misspliced transcript at the level of exon 15 of SPG4 complement DNA. Patient 3, in addition to the splice-site mutation, showed a heterozygous base change in position −4 of the consensus for the splice site c.1616-4 T/H11022 A (Table 2), which represents a frequent polymorphism (10 of 50 chromosomes).

Five novel mutations were detected outside the AAA domain of spastin: a duplication in exon 1, an insertion/deletion mutation in exon 6, 2 missense mutations (p.L195V and p.W607C), and a deletion in exon 2 (Table 2).

Duplication of 19 bp in exon 1 (c80-98dup19) was found in all affected members of family HSP1 (Figure 1A). This mutation, which leads to an early protein truncation
Molecular analysis of the SPG4 gene in this series of 60 patients with HSP led to the identification of 12 mutations in 13 patients (5 familial and 8 sporadic cases) with pure HSP. No mutations were found in patients with complicated forms of the disease. Thus, the overall frequency of SPG4 mutations in our study was 22% (13 of 60 patients), rising to 26% (13 of 50 patients) if we considered only patients affected by pure forms of HSP. This figure is consistent with what was already observed in other populations (5%-44%). 1,5,7,11 Eight of these mutations are novel, while 4 have already been described. 5,20 All 5 of the familial cases we analyzed had mutations in SPG4, confirming that the most common form of autosomal dominant HSP is caused by mutations in the SPG4 gene. The frequency of SPG4 mutations among the sporadic cases with pure HSP was 18% (8 of 45 patients). This figure is slightly higher than the one recently reported by Depienne et al. 20 (12%), possibly owing to the different type of population analyzed. The clinical data available for the patients in this study did not allow an easy genotype-phenotype correlation. In only 2 families (HSP5 and HSP4) could the mild clinical picture we observed be related to the conservative type of amino acid substitution detected (p.L195V and p.I406V); in both cases all residues are neutral and hydrophobic. The clinical variability observed in family HSP4 is likely due to the additional presence of an intragenic negative modifier, p.S44L, which in association with p.I406V might be responsible for the more severe phenotype and the anticipation observed in subjects III:1 and III:2. The genetic modifier p.S44L is a rare polymorphism within the Italian population (0.6%), as well as the North American population. 22 Moreover, the results of our study confirm that p.S44L cannot be the major determinant for HSP phenotype, as already reported. 22

Overall, in our patient sample, no correlation existed between patients carrying mutations within the AAA cassette and patients with mutations outside this domain. However, the growing number of mutations falling outside the AAA domain indicates that other important domains contribute to the functional role of spastin protein, such as the poorly studied C-terminal region, where we (in the present study) and others 6,23,24 have found mutations.

To summarize, our study described 8 novel mutations in both familial and sporadic cases of patients with pure HSP, thus expanding the mutational spectrum of the SPG4 gene. Molecular analysis of the SPG4 gene is advisable for sporadic cases of patients with HSP who present with spastic paraplegia of unknown cause. In addition, mutations in other genes responsible for dominant forms of the disease do not represent common causes of pure HSP. These findings might facilitate and better direct diagnostic tests in HSP.
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