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Spectrum of *SPG4* mutations in autosomal dominant spastic paraplegia

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Autosomal dominant hereditary spastic paraplegia (AD-HSP) is a group of genetically heterogeneous neurodegenerative disorders characterized by progressive spasticity of the lower limbs. Five AD-HSP loci have been mapped to chromosomes 14q, 2p, 15q, 8q and 12q. The *SPG4* locus at 2p21–p22 has been shown to account for ~40% of all AD-HSP families. *SPG4* encoding spastin, a putative nuclear AAA protein, has recently been identified. Here, sequence analysis of the 17 exons of *SPG4* in 87 unrelated AD-HSP patients has resulted in the detection of 34 novel mutations. These *SPG4* mutations are scattered along the coding region of the gene and include all types of DNA modification including missense (28%), nonsense (15%) and splice site point (26.5%) mutations as well as deletions (23%) and insertions (7.5%). The clinical analysis of the 238 mutation carriers revealed a high proportion of both asymptomatic carriers (14/238) and patients unaware of symptoms (45/238), and permitted the redefinition of this frequent form of AD-HSP.

INTRODUCTION

Hereditary spastic paraplegia (HSP) is a group of clinically and genetically heterogeneous neurodegenerative disorders mainly characterized by progressive and bilateral spasticity of the lower limbs. The major pathological features of HSP are degeneration of the crossed pyramidal tracts and thinning of the dorsal columns (1). HSP has been classified according to the mode of inheritance and the presence or absence of additional symptoms. It has been divided into two forms (2) depending on whether spasticity occurs in isolation (pure HSP) or associated with a wide range of additional clinical features (complicated HSP). Although both forms can be inherited in an autosomal dominant (AD-HSP), an autosomal recessive (AR-HSP) or an X-chromosome-linked (X-HSP) manner, pure spastic paraplegia is the most common form of AD-HSP. Two subgroups of AD-HSP have been distinguished according to the age at onset (3,4), one with an onset <35 years (type I) and the other with onset >35 years (type II). It was later suggested (5) that onset at 20 years of age might be a more discriminating cut-off point to avoid overlap between type I and type II. However, the remarkable variation in age at onset described both amongst and within families has indicated that this nosological criterion is not appropriate to subclassify HSP (6).

Pure AD-HSP has been shown to be genetically heterogeneous (7) and five loci have been identified to date on chromosomes 14q (*SPG3*) (7), 2p (*SPG4*) (8,9), 15q (*SPG6*) (10), 8q (*SPG8*) (11) and 12q (*SPG10*) (12). Approximately 40% of AD-HSP pedigrees show linkage to chromosome 2p, indicating the high prevalence of the *SPG4* locus in this form of the disease (13–15). After ruling out the possibility of a CAG repeat expansion in *SPG4* AD-HSP (16), we have recently isolated the gene underlying this form of AD-HSP using a positional cloning strategy based on the sequencing of the entire *SPG4* interval, and have identified five different mutations in seven families (17).

SPG4 (also known as *SPAST*; GenBank accession no. AJ246003) spans a physical distance of ~90 kb, is composed of 17 exons and encodes a putative nuclear member of the AAA [ATPases associated with diverse cellular activities (18,19)] protein family, named spastin (GenBank accession no. AJ246001) (17). The five DNA modifications (one nonsense, one

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Table 1. *SPG4* mutations in patients with AD-HSP

Family	Location	Mutation[a]	Amino acid change[b]	Consequence
624	Exon 7	1210C→G	S362C	Missense
6958	Exon 8	1233G→A	G370R	Missense
214	Exon 8	1267T→G	F381C	Missense
1002	Exon 8	1283T→G	N386K	Missense
027	Exon 8	1288A→G	K388R	Missense
019	Exon 10	1401C→G	L426V	Missense
4014	Exon 11	1468G→A	C448Y	Missense
148	Exon 11	1504G→T	R460L	Missense
618	Exon 13	1620C→T	R499C	Missense
636	Exon 15	1788G→A	D555N	Missense
627	Exon 15	1792C→T	A556V	Missense
2971	Exon 3	702C→T	Q193STOP	Nonsense
3655	Exon 5	873A→T	K229STOP	Nonsense
1010	Exon 5	907C→A	S261STOP	Nonsense
3938	Exon 5	932C→G	Y269STOP	Nonsense
6922	Exon 10	1416C→T	R431STOP	Nonsense
616	Exon 10	1416C→T	R431STOP	Nonsense
605	Exon 15	1809C→T	R562STOP	Nonsense
030	Exon 2	578–579insA	PTC + 2 aa	Frameshift
615	Exon 5	852del11	PTC + 18 aa	Frameshift
042	Exon 5	882–883insA	PTC + 12 aa	Frameshift
032	Exon 5	906delT	PTC + 17 aa	Frameshift
189	Exon 9	1299delG	PTC + 3 aa	Frameshift
018	Exon 9	1340del5	PTC + 35 aa	Frameshift
3686	Exon 9	1340del5	PTC + 35 aa	Frameshift
625	Exon 9	1340del5	PTC + 35 aa	Frameshift
A	Exon 11	1520delT	PTC + 1 aa	Frameshift
115	Exon 12	1574delGG	PTC + 2 aa	Frameshift
3266	Exon 13	1634del22	PTC + 18 aa	Frameshift
149	Exon 14	1684–1685insTT	PTC + 9 aa	Frameshift
645	Exon 14	1685del4	PTC + 7 aa	Frameshift
029	Intron 4	808–2a→g	?	Splicing mutation
162	Intron 6	1129+2t→g	?	Splicing mutation
125	Intron 7	1223+1g→t	?	Splicing mutation
143	Intron 8	1299+1g→a	?	Splicing mutation
1620	Intron 11	1538+5g→a	(PTC + 6 aa)	Exon 11 skipping + frameshift
1006	Intron 11	1538+3del4	?	Splicing mutation
1605	Intron 13	1661+1g→t	?	Splicing mutation
1012	Intron 13	1662–2a→t	?	Splicing mutation
1626	Intron 15	1812+1g→a	?	Splicing mutation
2992	Intron 15	1813–2a→g	Δaa564→aa576 (PTC + 7 aa)	Exon 16 skipping + frameshift
5226	Intron 15	1813–2a→g	Δaa564→aa576 (PTC + 7 aa)	Exon 16 skipping + frameshift
5330	Intron 15	1813–2a→g	Δaa564→aa576 (PTC + 7 aa)	Exon 16 skipping + frameshift
1611	Intron 16	1853+1g→a	?	Splicing mutation

[a]Nucleotide numbers refer to the SPG4 cDNA sequence. Bases in exons are denoted by upper case letters, bases in introns by lower case letters.
[b]Amino acid (aa) numbers refer to the spastin peptide sequence. PTC + *n* aa, premature termination codon at *n* amino acids downstream from the sequence alteration.

splice site and three missense mutations) detected so far in the seven AD-HSP families have unexpectedly suggested that this dominant degenerative disease results from a loss of function (17). In an attempt to establish phenotype–genotype correlations and to assess the frequency of *SPG4* mutations in AD-HSP, we have screened *SPG4* for mutations in 17 additional *SPG4*-linked AD-HSP kindreds as well as in 70 AD-HSP families with unknown linkage.

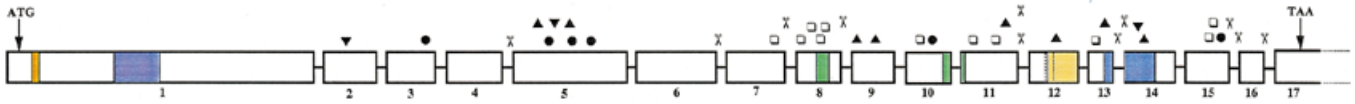

Figure 1. Schematic representation of the *SPG4* gene including the localization of the 39 mutations detected so far. The 17 exons of *SPG4* are drawn approximately to scale. The nucleic sequences encoding spastin conserved domains are indicated by different colours: the putative nuclear localization signal (amino acid position 7–11) in orange, the two leucine-zipper motifs (amino acid positions 50–78 and 508–529) in blue, Walker motifs A, the ATP-binding site, and B (amino acid positions 382–389 and 437–442, respectively) in green, the AAA minimal consensus (amino acid positions 480–498) in yellow and the helix–loop–helix dimerization domain (between amino acid positions 478 and 486) is framed by dashes in exon 12. Squares, missense mutations; circles, nonsense mutations; triangles, microdeletions; inverted triangles, insertions; scissors, splice site mutations.

RESULTS

SPG4 mutations in AD-HSP families

Two affected members and one control subject from each of the 17 *SPG4*-linked families and one affected individual from each of the 70 kindreds with unknown linkage were screened for mutations in *SPG4* using PCR amplification and sequence analysis of all 17 exons. A total of 39 different mutations, including the five DNA modifications described previously (17), were detected in 44 AD-HSP families (Table 1) originating from various geographical backgrounds (25 from France, 5 from Portugal, 3 each from Switzerland, Italy and Ireland, and 1 each from the UK, Germany, Spain, Poland and Tahiti). Mutations in *SPG4* co-segregate with the disease in 22 of the 24 *SPG4*-linked AD-HSP families and in 22 of the 70 AD-HSP kindreds with unknown linkage. Considering all the AD-HSP families that we studied either by linkage analysis or by mutation screening, our results suggest that *SPG4* HSP accounts for at least 37% of AD-HSP.

Two striking features are emphasized by this study. (i) All types of DNA modification including 9 deletions, 3 insertions and 27 base substitutions (11 missense, 6 nonsense and 10 splice site mutations) have been shown to affect *SPG4* in almost every exon or adjacent splice sites (Fig. 1). (ii) With the exception of three mutations (1416C→T, 1340del5 and 1813-2a→g) that were detected in more than one kindred, a different mutation co-segregates with the disease in each family. Amongst these 39 sequence alterations, 11 mutations (28%) lead to an amino acid substitution in the spastin sequence whereas at least 19 mutations (50%) result in a truncated protein (Table 1). Eleven splice site mutations including one deletion and ten base substitutions have been identified in our family sample, which represents ~28% of the *SPG4* mutations detected so far.

Missense mutations and spastin functional domains

Although these 39 mutations were found scattered along the length of *SPG4*, with the exception of exons 1, 4, 6 and 17, the 11 missense mutations are all localized between exons 7 and 16 (Fig. 1). This region of *SPG4* was shown to correspond to a highly conserved ATPase domain (17), also called the AAA cassette, that is predicted to harbor the ATPase activity in the AAA family members (20). Of the 11 missense mutations, eight lead to drastic amino acid changes including four substitutions involving cysteines, whereas the remaining three, 1288A→G (K388R), 1401C→G (L426V) and 1792C→T (A556V), result in conservative amino acid changes (Table 1). These three sequence alterations located in exons 8, 10 and 15, respectively, were not detected in 96–124 control chromosomes, indicating their mutation status. The presence of conservative amino acid changes may reveal the functional importance of such affected residues. Here, two missense mutations, 1283T→G (N386K) and 1288A→G (K388R), were shown to affect the spastin Walker motif A which corresponds to the ATP-binding domain of all ATPases (21). Interestingly, mutation 1283T→G results in a non-conservative amino acid change (N386K) at the non-conserved position 5 of the Walker minimal consensus motif A, GPPG**X**GKT (mutated amino acid in bold), whereas mutation 1288A→G generates a conservative change (K388R) at the extremely well conserved position 7 of Walker motif A. These combined data suggest that Lys388 is directly involved in binding ATP, and thus appears to be critical for spastin function.

Polymorphisms

Sequence analysis of all *SPG4* exons and exon–intron boundaries was carried out on 142 individuals (one unaffected and two affected members of the 24 *SPG4*-linked families and one patient from each of the 70 unlinked kindreds). Although this analysis revealed the presence of intronic single-nucleotide polymorphisms (SNPs), it failed to detect any SNPs in coding exons, including synonymous or conservative codon changes on non-carrier chromosomes and in the non-mutated regions of carrier chromosomes.

Clinical analysis of *SPG4* patients

A total of 238 mutation carriers including 179 patients aware of symptoms were identified in the 44 *SPG4* families. The phenotype was pure spastic paraplegia in 41 of these families and a complicated form of AD-HSP associated with cognitive signs was observed in three kindreds. The main clinical characteristics of the affected family members are shown in Table 2. A total of 45 patients (~20%) were unaware of symptoms at examination although 10 of them were definitely affected, 30 were probably affected and 5 had a possible spastic paraplegia. In addition, 14 asymptomatic carriers (mean age at examination 36 ± 18 years, range 19–73 years) were detected by this mutation screening. Mean age at onset was 29 ± 17 years, range 0 to 74 years, which is consistent with the adult onset determined for 61 patients from 12 *SPG4*-linked AD-HSP kindreds (29 ± 15 years) (13). However, the distribution of age at onset for the affected members of the 44 *SPG4* families (Fig. 2) has shown that 40% of the patients had onset before 30 years.

Table 2. Clinical characteristics of *SPG4* patients

No. of patients (women/men)	224 (105/119)
Mean age at onset in years (range) $n = 172$	29 ± 17 (0–74)
Mean disease duration in years (range) $n = 172$	21 ± 15 (0–73)
Mean age when independent walking was impossible	48 ± 17 (22–79)
No. of affected individuals unaware of symptoms	45 (20%)
Clinical features	
Severe spasticity at gait/at rest	34% (74/209)/19% (28/151)
Increased reflexes in the lower limbs	91% (195/214)
Increased reflexes in the upper limbs	27% (54/204)
Extensor plantar reflex	81% (174/216)
Ankle clonus	62% (87/141)
Proximal muscle weakness in the lower limbs	54% (113/208)
Distal muscle weakness in the lower limbs	36% (64/179)
Distal muscle wasting	9% (16/176)
Decreased vibration sense at ankles (abolished)	58% (111/208) (11%, 24/208)
Urinary urgency/incontinence	38% (74/197)/2% (4/197)
Pes cavus/scoliosis	21% (35/170)/5% (7/154)

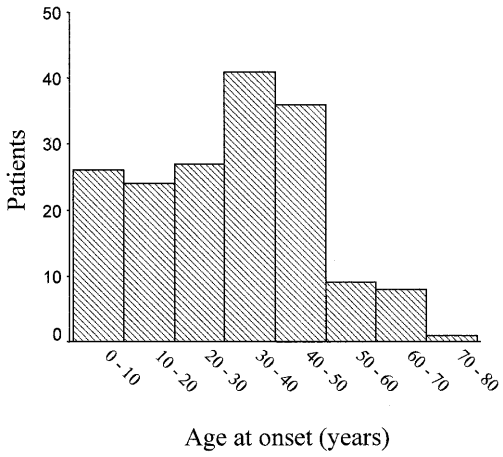

Figure 2. Distribution of age at onset in 172 *SPG4* patients.

The initial symptom was the insidious appearance of stiffness in the legs for 86% of the patients (130/151). Nineteen per cent of the affected family members (28/151) complained of gait unsteadiness or falls, and 5% (8/151) suffered from pain in the lower limbs. Pyramidal signs were variably associated with several clinical features that are often observed in pure spastic paraplegia, such as decreased vibration sense in the lower limbs (58%), urinary urgency (38%), pes cavus (21%) and scoliosis (5%). None had cerebellar gait ataxia but a mild cerebellar syndrome in the upper limbs was diagnosed in four patients. None had parkinsonian signs, dystonia or decrease in visual acuity. Brisk reflexes in the upper limbs were observed in 27% of the patients.

Disease severity was highly variable among patients (Table 3). We compared the frequency of clinical signs between four groups of patients showing increasing disease duration (Fig. 3). The presence of the different symptoms varied with disease duration: proximal lower limb weakness ($P < 0.001$), proximal and distal wasting ($P < 0.001$), decreased vibration sense in the lower limbs ($P < 0.001$), spasticity at rest ($P < 0.05$) and urinary urgency or incontinence ($P < 0.01$) were significantly more frequent with increasing disease duration (Fig. 3). As suggested by Harding (3), the phenotype of patients with an early onset (≤35 years) differed from that of affected individuals with a late onset (>35 years). Although the clinical sign frequency was similar between the two groups of patients, the disease progression determined by the severity index was significantly faster in the late onset group ($P < 0.001$) (Fig. 4), which confirms Harding's data (3).

Table 3. Mean disease duration according to disability stages

	No. of patients	Mean disease duration (years)[a]
Stage 1 (normal or very slight stiffness in the legs)	30	9 ± 8
Stage 2 (moderate gait stiffness)	28	12 ± 10
Stage 3 (unable to run but able to walk alone)	63	18 ± 9
Stage 4 (walk with help)	39	25 ± 11
Stage 5 (wheelchair-bound)	32	37 ± 16

[a]$P < 0.001$.

Penetrance was age dependent and incomplete even in older mutation carriers. A 73-year-old patient (from family 1611) with a splice site mutation was completely asymptomatic at examination, whereas the oldest affected individual unaware of symptoms but clinically affected was aged 62. Penetrance, that is defined as the proportion of symptomatic mutation carriers, increased with the age at examination, from 60% in the group of younger patients (10–19 years) to 76% in 20- to 40-year-old patients and 85% after 40 years.

Genotype–phenotype correlation

Comparison of the phenotypes observed in patients carrying a missense mutation ($n = 36$) versus patients with a truncated

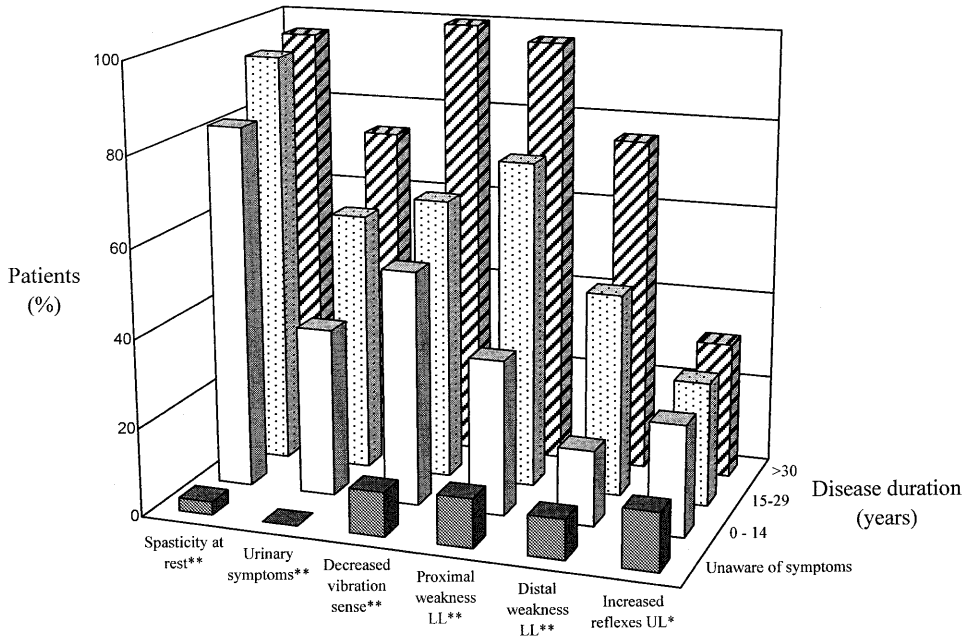

Figure 3. Evolution of clinical signs as a function of disease duration. Patients unaware of symptoms ($n = 45$), affected individuals with disease duration between 0 and 14 years ($n = 64$), between 15 and 29 years ($n = 72$) and >30 years ($n = 36$). LL, lower limbs, UL, upper limbs. The P values represent significant differences in each clinical sign between the four different groups of patients with increasing disease duration: $*P < 0.05$, $**P < 0.001$.

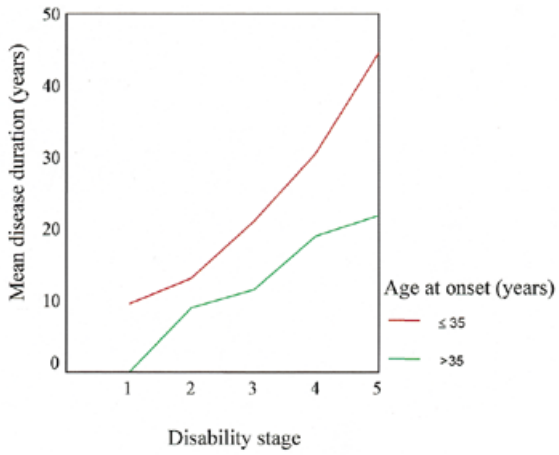

Figure 4. Disease progression as a function of disease duration for each disability stage in patients with onset before or after 35 years.

spastin ($n = 103$) failed to reveal any difference in the clinical sign frequency and the disease severity which was estimated by the mean disease duration in each disability stage. Moreover, we did not observe any significant difference in the age at onset between patients with a missense mutation and patients with a truncated protein (24 ± 18 versus 31 ± 17, $P = 0.08$). The phenotypes of patients carrying a conservative missense mutation (families 019, 027 and 627) are similar to those observed in patients with a non-conservative missense mutation or a truncated protein: clinical features were not less frequent or less severe in patients with conservative missense mutations, which tends to confirm the importance of the residues affected by these changes in spastin function.

DISCUSSION

SPG4, encoding a new member of the AAA protein family, has recently been identified as responsible for the most frequent form of AD-HSP (17). Five different heterozygous mutations located in the coding region of this gene have been found in seven AD-HSP families. In the present study, sequence analysis of the 17 exons of *SPG4* in 87 unrelated patients affected with AD-HSP has resulted in the detection of 34 novel mutations. Considering all the AD-HSP families that were investigated either by linkage analysis or by mutation screening, we may ascertain that *SPG4* AD-HSP accounts for 37% of all AD-HSP. The 39 *SPG4* mutations reported to date are scattered along the coding region of the gene and include missense (28%) and nonsense (15%) mutations, deletions (23%), insertions (7.5%) and splice site point mutations (26.5%). As suggested previously (17), all these types of DNA alteration indicate that this form of AD-HSP results from a loss of spastin function (22).

The splice site mutations, and the deletions or insertions generating a truncated protein are all deleterious since they seem to result in unstable aberrant transcripts and thus may lead to a decrease in spastin. Haploinsufficiency, though unexpected for an autosomal dominant neurodegenerative disorder, was observed in at least 50% of the *SPG4* AD-HSP families analyzed so far. On the other hand, all missense mutations are localized within the spastin functional domain, the aforementioned AAA cassette, and therefore might cause a loss of its activity. Since spastin belongs to the AAA protein family, it may be involved in the assembly, function or disassembly of protein complexes and thus act as a chaperone molecule (23). Mutations producing a loss of function either by inactivation of the functional domain or by a reduction in the amount of spastin might consequently cause a decrease in the

number of functional protein complexes that are crucial for axonal preservation in a subset of neurons. In this case, the abnormal phenotype could be due to the resulting imbalance with the matched proteins forming these complexes. However, the possibility that spastin normally works close to a threshold level cannot be ruled out. Both hypotheses could account for the important variability in severity and age at onset, and incomplete penetrance of *SPG4* AD-HSP.

As observed in other autosomal dominant and recessive disease-causing genes, such as *PAX6* responsible for aniridia (24) and *ATM* underlying ataxia-telangiectasia (25), most of the 39 *SPG4* mutations detected so far result in protein truncation whereas only 28% are missense mutations. Moreover, the frequency of *SPG4* splice site mutations (28%) is significantly higher than that reported in surveys of other human genetic disorders, in which 15% of mutations are found to affect mRNA splicing (26,27). Interestingly, no coding SNPs (cSNPs), including synonymous polymorphisms, could be identified in *SPG4* exons as described by Cargill *et al.* (28) for 13 of 106 genes analyzed in a systematic survey of cSNPs. The absence of cSNPs on non-carrier chromosomes and the presence of conservative missense mutations might suggest that any base substitution affecting the *SPG4* coding sequence is deleterious. However, the localization of the three conservative missense mutations within the AAA cassette tends to highlight the functional importance of some residues compared with others.

The clinical analysis of the 224 patients carrying a mutation in *SPG4* has led us to redefine this frequent form of AD-HSP. *SPG4* AD-HSP should no longer be considered only as an adult-onset form of HSP, contrary to what has previously been suggested (8,13), since 40% of the patients had onset before 30 years. However, the mean age at onset is later and the range of ages at onset is larger in *SPG4* AD-HSP than in other forms of HSP such as *SPG3*, *SPG5* or *SPG7*. Most patients displayed pyramidal signs (i.e. increased reflexes in the lower limbs, extensor plantar reflexes, ankle clonus and muscle weakness in the lower limbs) and decreased vibration sense at ankles. Other classical signs of pure HSP such as sphincter disturbances, distal muscle wasting in the lower limbs, pes cavus, scoliosis or mild cerebellar syndrome in the upper limbs were less frequent. These signs were also variably found in other forms of AD-HSP. Cognitive impairment was the only atypical symptom observed in 9 of 19 affected individuals from three kindreds. Disease severity was highly variable both within and among families: we observed asymptomatic mutation carriers >70 years old as well as severely affected patients who were wheelchair-bound in their third decade.

We have confirmed that the disease progression in patients with late-onset *SPG4* AD-HSP is significantly faster than in patients with early onset, which is consistent with Harding's conclusion on overall AD-HSP (3). Our results have also established that phenotype variability clearly depends on disease duration in *SPG4* AD-HSP. Both functional impairment measured on a five-point disability scale and the frequency of some clinical signs such as weakness, wasting and decreased vibration sense in the lower limbs, sphincter disturbances and spasticity at rest increase with disease duration. Comparison of the phenotypes between missense mutation carriers and patients with a truncated spastin did not reveal any significant difference in disease progression, symptom severity or age at onset. Although these findings need to be confirmed for other patient samples, they suggest that missense mutations are unlikely to exert a dominant-negative effect on spastin activity which should lead to a more severe phenotype than truncating mutations producing haploinsufficiency. Further clarification of the molecular mechanism underlying this neurodegenerative disorder should be provided by the creation of mouse models for *SPG4* AD-HSP with either a missense mutation or a truncated protein.

As demonstrated for *SPG7* mutations (29), both pure and complicated forms of AD-HSP are caused by *SPG4* mutations since two nonsense and one missense mutations co-segregate with the disease in families 2971, 1010 and 1002, which display a spastic paraplegia associated with cognitive signs. These findings suggest that the distinction between pure and complicated remains ambiguous. However, dementia was not present in all affected individuals (9/19) from these three families and no *SPG4* mutations were detected in other families with complicated AD-HSP. Therefore, pure AD-HSP remains so far the most frequent presentation of spastin defects. Considering the extended genetic heterog geneity of AD-HSP, a more precise nosological criterion should be based on the different loci responsible for HSP.

The first application of the identification of a disease-causing gene is to establish pre- and postnatal molecular diagnosis of the disorder. It seems now obvious that accurate diagnosis of *SPG4* AD-HSP can only be obtained by mutation analysis because of the high proportion of both asymptomatic mutation carriers and patients unaware of symptoms. If the latter patients can be detected by a detailed clinical examination, asymptomatic carriers can only be identified by molecular analyses. This observation and the marked intrafamilial variability in severity make genetic counselling for prenatal diagnosis and presymptomatic testing particularly delicate.

MATERIALS AND METHODS

Patients and statistical analysis

In addition to previously reported *SPG4*-linked families (618, 624, 625, 627, 645, 4014, A, 2992, 5330, 5226, 1620, 3266, 2971 and 1001) (13,30,31), 80 families with progressive spastic paraplegia were recruited using the following criteria: presence of at least two patients with clinically definite spastic paraplegia and transmission of the disease in at least two successive generations. Patients were classified as having: (i) definite spastic paraplegia, i.e. spasticity, increased reflexes and extensor plantar response; (ii) probable spastic paraplegia, i.e. only increased reflexes or extensor plantar response; and (iii) possible spastic paraplegia, i.e. brisker reflexes in the lower limbs compared with the upper limbs. Onset was defined as the year of the first symptoms according to the patient. Complicated forms of spastic paraplegia segregate in five of these families since additional clinical signs, such as dementia in four families (2971, 1001, 1002 and 1010), cerebellar syndrome in kindred 610 and peripheral neuropathy in family 627, were diagnosed in at least two affected family members. Disability was assessed on a five-point scale: 1, normal gait or very slight stiffness in the legs; 2, moderate gait stiffness; 3, unable to run but able to walk alone; 4, walk with help;

5, wheelchair-bound. Informed and written consent was obtained from each individual.

Statistical significance was determined using the χ^2 test with Yates' correction when appropriate, and non-parametric Mann-Whitney *U* comparison. Since the families were selected for the presence of at least two affected members in two generations, these individuals were excluded from the penetrance estimate to take into account the selection bias.

Mutation screening

The 17 coding exons of *SPG4* were amplified by PCR from 100 ng of genomic DNA and sequenced on an ABI 377 sequencer (PE Applied Biosystems, Les Ulis, France) as previously described (17). Total RNA was extracted from patient lymphoblastoid cell lines when available using the RNA PLUS kit (Bioprobe System; Quantum-Aplligene, Illkirch, France) and cDNA synthesis was performed on ~500 ng to 1 µg of each RNA sample with 100 pmol of random primers (Amersham Pharmacia Biotech, Saclay, France) and 200 U of Superscript II RT (Gibco BRL, Life Technologies, Rockville, MD) according to standard procedures. Four PCR amplifications which generated overlapping fragments spanning the *SPG4* open reading frame were carried out on the patient cDNAs and all PCR products were sequenced on an ABI 377 sequencer (PE Applied Biosystems) as reported previously (17). All primers used to amplify and sequence both *SPG4* exons and *SPG4* cDNA are available at http://www.genoscope.cns.fr . When sequence analysis revealed a nucleotide variation in a patient, its co-segregation with the disease was then ascertained in the rest of the family. Each of the three exons, 8, 10 and 15, in which conservative mutations were identified, were examined in 48–62 controls selected from the CEPH families.

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