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# Expansion of mutation spectrum, determination of mutation cluster regions and predictive structural classification of *SPAST* mutations in hereditary spastic paraplegia

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The *SPAST* gene encoding for spastin plays a central role in the genetically heterogeneous group of diseases termed hereditary spastic paraplegia (HSP). In this study, we attempted to expand and refine the genetic and phenotypic characteristics of *SPAST* associated HSP by examining a large cohort of HSP patients/families. Screening of 200 unrelated HSP cases for mutations in the *SPAST* gene led to detection of 57 mutations (28.5%), of which 47 were distinct and 29 were novel mutations. The distribution analysis of known *SPAST* mutations over the structural domains of spastin led to the identification of several regions where the mutations were clustered. Mainly, the clustering was observed in the AAA (ATPases associated with diverse cellular activities) domain; however, significant clustering was also observed in the MIT (microtubule interacting and trafficking), MTBD (microtubule-binding domain) and an N-terminal region (228–269 residues). Furthermore, we used a previously generated structural model of spastin as a framework to classify the missense mutations in the AAA domain from the HSP patients into different structural/functional groups. Our data also suggest a tentative genotype–phenotype correlation and indicate that the missense mutations could cause an earlier onset of the disease.

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## Introduction

Hereditary spastic paraplegias (HSPs), also known as the Strümpell–Lorrain syndrome, are a group of neurodegenerative disorders caused by monogenic mutations. The universal clinical feature of the disease is progressive bilateral weakness and spasticity of the lower limbs.<sup>1</sup>

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Histopathological studies of HSP patients revealed selective degeneration of some of the longest axons. The degenerative process initially affects the distal ends of these axons and then proceeds proximally toward the cell body.<sup>2,3</sup> On the basis of clinical features, HSP can be classified into two forms: the pure and the complex HSP. In pure HSP, spasticity occurs in relative isolation; however, when additional neurological or non-neurological symptoms are associated with spasticity then it is termed as complex HSP.<sup>4,5</sup>

HSPs are genetically heterogeneous and different modes of inheritance are reported, including autosomal dominant, recessive and X-linked HSP; within each inheritance group, there is further locus heterogeneity. Fifteen of the

HSP genes have been identified. However, mutations in the *SPAST* gene (MIM 604277) encoding for spastin protein is the most common cause of HSP<sup>6</sup> and accounts for 15–40% of all AD-HSP cases, depending upon the ethnic origin of the selected cohort of patients.<sup>6–12</sup> The prevalence of *SPAST* mutations in sporadic cases and with uncertain family history is much lower (12–18%).<sup>13,14</sup>

Spastin belongs to the AAA (ATPases associated with diverse cellular activities) family of proteins.<sup>15–17</sup> The AAA proteins are proven or putative ATPases and they are characterized by a conserved C-terminal domain containing one or two AAA cassettes.<sup>15</sup> Apart from the AAA domain, these proteins consist of various other domains, which interact with adapter proteins to generate the structural and functional diversity of the family.<sup>16,18</sup>

Screening for mutations in the *SPAST* gene by various groups has identified over 224 different mutations in most exons except for exon 4, which is alternatively spliced. The *SPAST* mutations are summarized in the Human Gene Mutation Database Professional release 7.1 (<http://www.biobase.de/hgmd/pro/start.php>). Different types of DNA alterations are detected in the *SPAST* gene, including missense, nonsense, splice site mutations and insertions/deletions. Recent studies used a *SPAST*-specific multiplex ligation-dependent probe amplification (MLPA) assay to demonstrate that a large proportion (18–20%) of patients with unlinked HSP, in fact, carry large deletions in the *SPAST* gene.<sup>19,20</sup> These findings further broaden the spectrum of the *SPAST* mutations.

From a diagnostic point of view, unfortunately, there are no common mutations in *SPAST*, with most families having private mutations. Moreover, there are neither obvious genotype–phenotype correlations between different types of spastin mutations, nor any hot spot regions for *SPAST* mutations have been identified. Therefore, screening of the complete coding sequence of *SPAST* is necessary for the detection of mutations.

## Materials and methods

### HSP cases/families

Blood samples from 200 unrelated HSP individuals/families, primarily from Germany were referred to Goettingen Molecular Genetics Service for diagnostic testing of the *SPAST* gene. Clinical data of these patients were obtained by clinical evaluations from the referring neurologist. All probands were selected on the basis of Harding's accepted criteria for the definition of the clinical status of HSP.<sup>21</sup> For ascertaining, a patient as HSP case following clinical features were assessed/observed in the lower limbs (lower extremities) of the patient; gait disorder owing to spastic paraparesis, spastic hypertonia, positive Babinski sign, ankle clonus, hyperreflexia, reduced pallesthesia and paresis. In addition, for complex HSP, cognitive deficits and other neurological/non-neurological symptoms were

also considered. The age at onset (AAO) was determined by the referring neurologist after clinical evaluation and consulting with the patient. If required, additional family members were also consulted to corroborate the AAO. In our HSP cohort, 99 cases showed familial inheritance, 46 sporadic cases and in 55 cases, we could not ascertain the mode of inheritance. In this cohort, there were 109 pure HSP, 23 complex HSP and 68 unknown cases. Informed consent was obtained from all probands.

### Mutation screening and detection

Genomic DNA from peripheral blood leukocytes of patients was isolated using standard procedures. The 17 exons of the *SPAST* gene (accession no. NM\_014946.3) and flanking intronic sequences were amplified by PCR. Primer sequences and PCR conditions are available on request. Purification of PCR products was performed using a PCR purification kit (Millipore). The purified PCR products were sequenced using both forward and reverse primers (which were used for the PCR amplification) using ET reaction kit (Amersham Biosciences) on a MegaBACE 500 sequencer (Amersham Biosciences). Nucleotide variations revealed by first sequencing reaction were verified by second independent PCR and sequencing reaction. All sequence variants reported here were checked in a panel of 50 healthy unrelated subjects recruited randomly from the German population.

### MLPA analysis

MLPA was performed with 200 ng of genomic DNA according to manufacturer's instructions using the P165 Salsa MLPA HSP probe set (MRC-Holland). Probe amplification products were run on an ABI 3130 DNA Analyzer using Liz600 size standard (Applied Biosystems). MLPA peak plots were visualized, normalized and the dosage ratios were calculated by using GeneMarker Software v1.51 (Soft Genetics LLC). Owing to variation in each assay performance, we used dosage ratio values of  $\leq 0.7$  and  $\geq 1.35$  as our boundaries for deletions and duplications, respectively.

### Detection of break points for small insertions and deletions

To determine the precise breakpoints of small insertions and deletions in the *SPAST* gene, the PCR amplicons were cloned into pGEMT Easy vector (Promega) according to the manufacturer's instructions. Plasmid DNA from at least 10 independent bacterial colonies were sequenced in both directions using vector-specific primers (T7 and SP6) using standard protocol.

### Modeling of the AAA domain of spastin

The AAA ATPase domain of spastin was modeled on the basis of the tertiary structures of two templates (PDB codes: 1xwi and 1s3s) using MODELLER Version 8.0 program.<sup>22</sup>

The generated models were energy-minimized using the Kollman united atom force field in SYBYL (Tripos Inc.) to ensure acceptable geometry and to relieve short contacts. The overall fit of the sequence to the template was checked using Verify 3D.<sup>22</sup> The copies of modeled tertiary structures were assembled to form a hexameric quaternary assembly on the basis of the hexameric template (1s3s). This modeled quaternary structure was energy-minimized using SYBYL.<sup>22</sup> The interfacial residues between the monomers were extracted using the Contacts of Structural Units program<sup>22</sup> and the crucial residues were short listed by manual inspection and used for further analysis.

### Labeling of mutated missense residues in the structural model of spastin

We used a previously generated structural model of spastin,<sup>22</sup> for labeling of the missense mutations identified in the AAA domain in our HSP cohort. In brief, the tertiary structure of the ATPase domain of spastin was modeled on the basis of the tertiary structures of two templates (PDB codes: 1xwi and 1s3s) and the quaternary structure was modeled on the basis of the hexameric template (1s3s). The consensus sequence/motifs in the AAA domain of spastin were structurally classified as active site, pore loop, protomer interacting and other residues and labeled as colored ribbons in the modeled tertiary structure. The novel spastin missense mutations identified in this study were labeled as colored space-fill structures in tertiary and quaternary structures using RasWin Molecular Graphics Version 2.7.3. (<http://www.rasmol.org/>).

The models of spastin are deposited in Protein Model Data Base (<http://mi.caspu.it/PMDB/>), which can be downloaded as PDB files (PDB codes: PM0074982 and PM0074984). The amino-acid residues in the spastin model are numbered from 1–286 aa, which corresponds to 331–616 aa of the full-length spastin isoform. To determine in which functional/structural group (active site, pore loop structure, protomer interface residues and other mutations) of spastin a novel sequence variant can be cataloged, the mutated residue can be labeled by using RasWin Molecular Graphics Version 2.7.3 program.

## Results

### Detection of SPAST mutations in the HSP cases by sequencing and MLPA analysis

A total of 200 DNA samples of unrelated HSP individuals/families were first screened for mutations in the SPAST gene by sequencing all the 17 exons of the gene, which led to the identification of 51 (25.5%) heterozygous mutations (Table 1). Next, to detect SPAST deletion, we performed MLPA analysis in the 149 HSP cases, which were identified as SPAST negative through sequence analysis. By MLPA analysis, we identified six (three of which represented novel mutations) additional large heterozygous deletions

in the SPAST gene, which accounts for 4% of the 149 HSP cases (Table 1). In our screen, 47 different mutations were detected and 29 of these mutations were novel (Table 1). None of the 29 identified novel mutations in our study were found in the 100 control chromosomes. In the analyzed HSP cohort, the mutation rate in the SPAST gene in pure HSP was 29.4% (32/109), in complex HSP it was 21.7% (5/23) and in cases of unknown clinical course the proportion was 29.4% (20/68) (Table S1). Furthermore, in familial HSP we observed a mutation frequency of 36.4% (36/99), in sporadic HSP, it was 6.5% (3/46) and in HSP cases with unknown inheritance pattern, we detected a mutation rate of 32.7% (18/55) (Table S1, Note: Table S1–S4 and Figure S1 are available as (online Supplementary Information)).

### Mutational spectrum and cluster regions in spastin

The distribution analysis of the 26 novel mutations (excluding the gross deletions) along the structural domains of spastin revealed that 22 (85%) mutations are localized in the conserved AAA domain, two (8%) mutations were in the MIT domain (Figure 1a). Owing to this skewed spectrum of mutations in the spastin, we included other spastin mutations reported in the database (<http://www.biobase.de/hgmd/pro/start.php>) in our analysis. Interestingly, we could identify several regions within the structural architecture of spastin where mutations were clustered (Figure 1b). The primary region was in the AAA domain (342–599 aa), which accounts for 72.7% of identified mutations in spastin (Figure 1b and Table S2). Additional secondary cluster regions, were detected in the MIT domain (116–197 aa) with 7.2% mutations, MTBD (microtubule-binding domain; 270–328 aa)<sup>30</sup> with 5.1% and an N-terminal region (228–269 residues) with 7.6% mutations (Figure 1b and Table S2). Overall, these four regions account for 92.7% of the mutations reported to date in spastin.

### Functional/structural classification of the missense mutations

Out of the 29 novel mutations, which we identified in our screen, 10 (38%) are missense mutations, which are located in the AAA domain. We attempted to classify these 10 missense mutations identified in the AAA domain into different structural/functional groups based upon a previously generated structural model of spastin (PMBD id: PM0074982 and PM0074984).<sup>22</sup> We could consign all these missense mutations in the three dimensional space of the spastin structure into four categories namely, active site, pore loop structure, protomer interface residues and other mutations (Table S3 and Figure S1A–K).

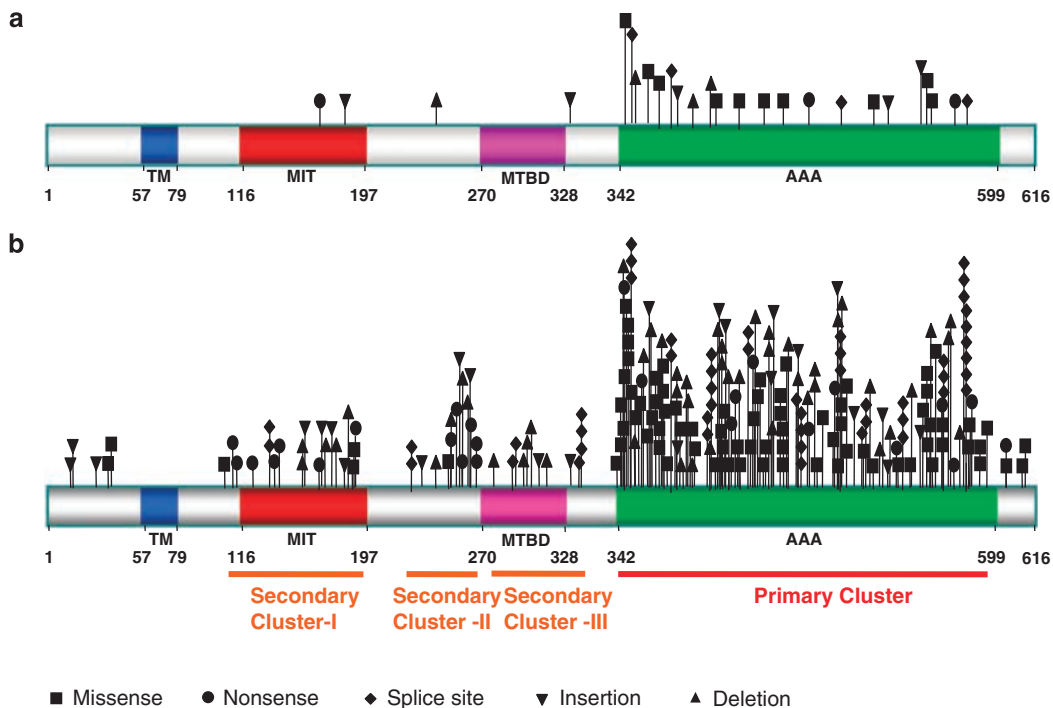
### S44L polymorphism in HSP

Co-inheritance of a disease-causing mutation and the L44 allele causes an early onset of symptoms, indicating that

**Table 1** List of mutations identified in the SPAST gene in our HSP cohort

No.	Exon/Intron	Family no.	Type of mutation	cDNA	Protein	F/S	Onset of phenotype	Pure/complex	Reference/novel
1	Ex 1	24227	Nonsense	c.373G>T	p.Glu125Term	F	>35	Pure	11
2	Ex 2	25942	Nonsense	c.499C>T	p.Gln167Term	S	>35	Pure	Novel
3	Ex 3	24283	Insertion	c.549_550insT	p.Asn184Term	F	<35	Pure	Novel
4	Ex 5	21987	Deletion	c.692delC	p.Ala231Valfs Term239	?	?	?	Novel
5	Ex 5	19583	Deletion	c.839_840delAG	p.Gln280Argfs Term289	F	<35	Pure	12
6	Ex 5	28146	Splice	c.870 G>A	Unknown	?	?	?	Novel
7	Int 5	25961	Splice	c.870+1G>T	Unknown	?	?	?	Novel
8	Ex 6	24295	Insertion	c.981_982insAT	p.Ile328Ilefs	F	<35	Complicated (cardio-myopathy)	Novel
9	Ex 7	21937	Missense	c.1067A>G	p.Glu356Gly	?	<10	?	6
10	Ex 7	21935	Missense	c.1081C>T	p.Pro361Ser	F	<35	Pure	Novel
11	Int 7	21971	Splice/deletion	c.1099-3_1099-1delTAG	Unknown	F	>35	Pure	Novel
12	Ex 8	21977	Deletion	c.1101_1103delGTT	p.Leu367del	F	>35	Pure	Novel
13	Ex 8	25902	Deletion	c.1101_1103delGTT	p.Leu367del	F	<10	Pure	Novel (2)
14	Ex 8	24218	Insertion	c.1115_1116insG	p.Arg372Argfs Term393	?	?	?	Novel
15	Ex 8	25945	Missense	c.1121C>G	p.Pro374Arg	F	<10	?	Novel
16	Ex 8	25946	Missense	c.1154G>A	p.Gly385Glu	S	>35	Pure	Novel
17	Int 8	24292	Splice	c.1174-1G>T	Unknown	F	<35	Pure	Novel
18	Ex 9	21938	Missense	c.1196C>T	p.Ser399Leu	F	?	?	23
19	Ex 9	24224	Missense	c.1196C>T	p.Ser399Leu	?	?	?	23
20	Ex 9	19576	Deletion	c.1202delC	p.Ala401Gluf- sTerm406	?	?	?	Novel
21	Ex 9	24286	Deletion	c.1215_1219delTATAA	p.Asn405Lysf- sTerm440	S	>35	Pure	8
22	Ex 9	24268	Missense	c.1216A>G	p.Ile406Val	F	<35	Pure	24
23	Ex 9	21901	Deletion	c.1245delC	p.Tyr415Term	S	>35	Pure	Novel
24	Int 9	24248	Splice	c.1245+1G>T	Unknown	F	<10	Pure	25
25	Ex 10	24231	Missense	c.1250G>A	p.Gly417Glu	F	<35	Complicated (cognitive impairment)	Novel
26	Ex 10	19582	Missense	c.1280T>G	p.Phe427Cys	F	>35	Pure	Novel
27	Ex 10	24233	Deletion	c.1281delT	p.Phe427Leufs Term437	?	?	?	26
28	Ex 10	24212	Nonsense	c.1291C>T	p.Arg431Term	F	>35	Pure	8
29	Ex 11	19593	Missense	c.1339T>G	p.Leu447Val	F	>35	Pure	Novel
30	Ex 11	21214	Missense	c.1378.C>T	p.Arg460Cys	F	>35	Complicated (polyneuro-pathay)	38
31	Ex 11	24222	Missense	c.1378C>T	p.Arg460Cys	?	<10	?	27
32	Ex 11	24285	Missense	c.1378C>T	p.R460C	F	<35	Pure	Novel
33	Ex 11	24228	Missense	c.1379G>A	p.Arg460Cys	F	<35	Pure	Novel
34	Ex 12	25910	Nonsense	c.1417C>T	p.Arg460His	F	<10	Autonomic nervous system	Novel
35	Ex 12	24255	Insertion	c.1462_1463insTA	p.Gln473Term	F	>35	Pure	Novel
36	Ex 13	25923	Missense	c.1495C>T	p.Arg488Ilefs sTerm530	F	<10	Pure	7
37	Ex 13	21929	Missense	c.1496G>A	p.Arg499Cys	F	<10	Trunk-ataxia	28
38	Ex 13	19598	Missense	c.1507C>T	p.Arg499His	?	?	?	14
39	Ex 14	21900	Missense	c.1540A>G	p.Arg503Trp	F	?	?	Novel
40	Ex 14	21985	Missense	c.1540A>G	p.Arg514Gly	F	>35	?	Novel (2)
41	Ex 15	24230	Insertion	c.1649_1650insCCTAAC	p.Arg514Gly	F	<35	Pure	Novel
42	Ex 15	19591	Missense	c.1664A>G	p.550_551in- sLeuThr	F	<35	Pure	Novel
43	Ex 15	25941	Missense	c.1670C>T	p.Asp555Gly	F	<35	Pure	Novel
44	Ex 15	21920	Nonsense	c.1684C>T	p.Ala557Val	?	?	?	8
45	Ex 15	21967	Nonsense	c.1684C>T	p.Arg562Term	?	>35	Pure	8
46	Ex 15	21974	Nonsense	c.1684C>T	p.Arg562Term	F	>35	Pure	8
47	Ex 15	24201	Nonsense	c.1684C>T	p.Arg562Term	F	<35	Pure	8
48	Ex 15	25912	Nonsense	c.1684C>T	p.Arg562Term	F	>35	Pure	8
49	Ex 16	19594	Nonsense	c.1702C>T	p.Arg562Term	?	?	?	Novel
50	Ex 17	19597	Missense	c.1821G>C	p.Gln568Term	?	?	?	13
51	Ex 17	25936	Missense	c.1821G>C	p.Trp607Cys	F	<35	Pure	13
52	Ex 1- Ex 3	24278	Exon deletion	c.1-?_682+?del	Unknown	?	?	?	19
53	Ex 2- Ex 9	21976	Exon deletion	c.416-?_1493+?del	Unknown	F	<35	Pure	Novel
54	Ex 8	21968	Exon deletion	c.1099-?_1173+?del	Unknown	F	<10	Pure	Novel
55	Ex 9 - Ex 17	21940	Exon deletion	c.1174-?_1851+?del	Unknown	F	>35	Pure	Novel
56	Ex 2 - Ex16	24270	Exon deletion	c.416-?_1728+?del	Unknown	F	>35	Pure	29
57	Ex 17	24281	Exon deletion	c.1729-?_18511?del	Unknown	?	?	?	19

Note: F, Familial; S, Sporadic.



TM – Transmembrane Domain; MIT – Microtubule Interacting and Trafficking Domain

MTBD – Microtubule Binding Domain; AAA – ATPase Associated with Various Cellular Activities Domain

**Figure 1** Schematic diagram representing the structural domains of the spastin protein (616 amino acids) and showing the localization of the identified mutations. The different domains are highlighted with different colors and different kinds of mutations are marked with different symbols as summarized on the left at the bottom of the figure. (a) Representation of 26 novel mutations identified in our study. Twenty-two out of 26 mutations are located in the AAA domain and two others in the MIT domain. (b) Distribution of all mutations identified to date in the spastin protein. The primary hot spot region is indicated as a red bar in the AAA domain. Three secondary hot spot regions are shown as orange bars below the spastin structure. Note: Three novel gross deletions are not included for representation; also gross deletions are excluded for distribution analysis.

the L44 allele is a genetic modifier of the HSP disease.<sup>10,31</sup> In our cohort of 200 HSP patients, the S44L variant was identified in six unrelated HSP cases in heterozygous state, therefore the incidence of the L44 allele in our cohort is 1.5%. In addition, in two HSP individuals, we detected a second mutation in *SPAST* apart from the L44 allele (Table S4).

### Putative genotype–phenotype correlations

To determine whether the spastin missense mutations have a different pathomechanism in contrast to other mutation types is to examine a correlation between mutation types and clinical features, such as age at onset of the symptoms. We therefore grouped the 57 identified mutations in our HSP cohort into two groups: the missense mutations (23) and the other types of mutations (34). We placed the number of identified mutations into two categories based upon age at onset of the symptoms, namely, onset before/ at 35 years ( $\leq 35$ ) and after 35 years ( $> 35$ ) as represented in

**Table 2** The proportions of missense and other types of mutations in different age groups, pure/complex and familial/sporadic cases of HSP

	Missense mutations	Other mutations	All mutations
< 35 years	23	34	57
> 35 years	13	13	26
Unknown	3	10	13
	7	11	18
Pure	11	21	32
Complex	3	2	5
Unknown	9	11	20
Familial	16	20	36
Sporadic	1	2	3
Unknown	6	12	18

Table 2. In the  $\leq 35$  age group, we observed an equal proportion of missense mutations (13/26; 50%) and other types of mutations (13/26; 50%) (Table 2). In the  $> 35$  age

group, we detected a higher fraction of other mutation types, (10/13; 76.9%) as compared with missense mutations (3/13; 23%) (Table 2). However, statistically the differences between the proportion of missense and other mutations in >35 age group was not significant ( $P>0.05$ ). Interestingly, we noticed a skewed distribution of missense mutations between the two age groups;  $\leq 35$  and  $>35$  years. Therefore, we tested a null hypothesis that there is no difference in the proportion of the subjects with missense mutations between two age groups  $\leq 35$  and  $>35$  years, the chi-square test demonstrated that there is a significant difference ( $P<0.012$ ), whereas no significant difference could be detected for subjects with other mutation types ( $P>0.5$ ).

## Discussion

In our current endeavor, we attempted to expand the mutational spectrum of *SPAST*. To this end, we screened for mutations in the *SPAST* gene in 200 HSP patients and identified 47 different mutations, out of which 29 were novel mutations. The overall frequency of *SPAST* mutations in our cohort was 28.5% (57/200). The mutation rate did not change significantly when we only considered pure HSP, which was 29.4% (32/109). Interestingly, in case of complex HSP a high mutational rate of 21.7% (5/23) was detected in our HSP cohort, which highlights the need to screen for the *SPAST* mutation in complex HSP cases. However, owing to lack of family history, we were unable to show segregation of additional symptom(s) with paraplegia, therefore, it is also possible that the complex phenotype could also be because of an independent locus other than the *SPAST*. The mutation detection rate in our cohort is consistent with the range of 15–44%, which was observed previously in other populations.<sup>8–11,13</sup> The frequency of mutations increased if we only considered the autosomal dominant HSP cases (36.4%) suggesting that prevalence of *SPAST* mutation is higher in the familial cases. Among the sporadic cases of HSP, the frequency of mutations was 6.5% (3/46), which was lower than the previously reported rate of 12–18%.<sup>13,14</sup> This discrepancy could be because of the different population type and size. Nevertheless, from a diagnostic point of view, our and other reports emphasize the need to screen for *SPAST* mutations in the sporadic HSP cases.

In our HSP cohort, we identified 51 mutations in the *SPAST* gene by direct sequencing of all the 200 HSP patients, which left 149 HSP cases in which no mutations could be detected by conventional sequencing. To determine gross deletion/insertion in the *SPAST* gene, we performed MLPA analysis in these 149 HSP cases and detected six additional mutations, which accounts for 4% (6/149) of remaining HSP cases. Previously, two independent studies used same *SPAST*-specific MLPA assay and

reported a much higher proportion (18–20%) of deletion in HSP patients.<sup>19,20</sup> The observed disparity in the proportion of large deletions between our HSP cohort and others could be because of the divergence and ethnic variability in these cohorts. Nevertheless, our report of much lower proportion of gross deletions in the *SPAST* gene in the HSP patients stresses the need to perform MLPA in various HSP cohorts to determine the incidence rate of gross deletions in worldwide HSP populations.

It is remarkable that 22 (85%) out of the 26 novel mutations (excluding the gross deletions) were located in the AAA domain of spastin. Previously, our group reported clustering of mutations in the AAA domain of spastin in a German HSP cohort<sup>12</sup> and this clustering in AAA domain was also observed in several other HSP cohorts.<sup>6–10</sup> Moreover, the distribution of mutations reported in the database over the structural domains of spastin outside the AAA domain were also not uniform; rather they were concentrated in certain regions of the protein, which constituted various functional domains, such as MIT and MTBD. In prior studies, exon 1, exon 5 and exon 8 of *SPAST* was recognized as hot spot regions,<sup>32–34</sup> however, no correlation to functional domain of spastin was implicated. Overall, it appears that different functional domains of spastin are target regions for mutations, which underlines their functional significance. Identification of these cluster regions highlights the need to set these regions as priority in the molecular diagnostic screens.

Beside a few exceptions, almost all the missense mutations in spastin are located in the AAA domain and recent studies suggest that these missense mutations might exert a dominant-negative effect on the molecular function of spastin.<sup>35,36</sup> Utilization of a recently modeled structure of the AAA domain of spastin,<sup>22</sup> as a framework, enabled us to classify the identified missense mutations from our cohort into different functional groups such as active site, protomer–protomer interaction, pore loop and unknown structural group of mutations. The functional categorization of the novel missense mutations, based upon the structural model of spastin will enable us in future to predict any identified sequence variant in a HSP-*SPAST* patient as disease-causing mutation with greater level of certainty. These structural predictions of various functional classes of missense mutations need to be validated by biochemical/cellular studies and data from the structural model should be interpreted with cautiousness. However, in a recent study, we could validate at the cellular level the functional effect of two sequence variants (E442Q and R499C) of spastin, which were predicted as active site mutations from the structural model of spastin.<sup>22</sup>

The rare S44L polymorphism is considered to act as a modifier of the HSP phenotype.<sup>10,31,37</sup> S44L is not considered as a susceptibility factor for HSP because its

frequency rate is similar in HSP patients and controls.<sup>14</sup> In our study, we could not ascertain the role of S44L (heterozygous state alone) on manifestation of HSP. It is possible that the patients heterozygous for S44L might have another mutation in spastin, which could not be identified by our screen or might have a mutation in a different HSP gene.

No apparent genotype–phenotype correlation is evident among missense mutations and other *SPAST* mutations.<sup>8,14,38</sup> Although several studies indicated that missense mutation might act in a dominant-negative fashion in contrast to other mutations, which lead to a loss of function. To determine, whether missense mutation leads to early onset of HSP, we assorted our HSP cohort into two different groups based upon AAO ( $\leq 35$  and  $> 35$  years). The rationale behind sorting our HSP cohort into these two age groups was derived from Harding's classification of HSP patients into two distinct groups, early age onset ( $\leq 35$  years) and late age onset ( $> 35$  years).<sup>39</sup> This AAO ( $\leq 35$  and  $> 35$  years) classification was also used by Fonknechten and coworkers for determination of genotype–phenotype correlation.<sup>8</sup> We observed an obvious difference in the proportion of mutations between the missense group as compared with the other types of mutations in age group of  $> 35$  years. However, the observed difference was not statistically significant ( $P > 0.05$ ) because of a very small sample size. Remarkably, we could reject a null hypothesis that there is no difference in the proportion of subjects for missense mutations between two age groups,  $\leq 35$  and  $> 35$  years, which was statistically significant ( $P < 0.0124$ ). Our data show a tentative genotype–phenotype correlation and suggest that in case of missense mutations the onset of phenotype is earlier. Owing to a small sample size, this correlation between AAO and missense mutation should be interpreted with discretion. Previously, early AAO in patients with missense mutation was also reported; however, this study only accounted for two missense mutations out of a total five mutations.<sup>40</sup> Moreover, in a meta-analysis<sup>38</sup> no significant correlation between AAO and mutational class was evident, but one limitation of this study was the sample size. Nevertheless, these different pathomechanism modes, such as loss of function and dominant-negative function for different classes/types of spastin mutations need to be carefully resolved by experimental means; otherwise there will be repercussions on the likely success of any therapeutical approach devised for spastin-associated HSP.

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