High frequency of partial SPAST deletions in autosomal dominant hereditary spastic paraplegia

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Background: Hereditary spastic paraplegia (HSP) is a genetically heterogeneous neurodegenerative disease. The most frequent cause of autosomal dominant HSP is mutation of SPAST (SPG4 locus), but additional pedigrees remain mutation negative by conventional screening despite linkage to SPG4. Objective: To determine the frequency of genomic copy number aberrations in autosomal dominant HSP. Methods: We developed and validated a multiplex ligation-dependent probe amplification assay targeting SPAST and SPG3A, another gene frequently involved in autosomal dominant HSP. In a multicenter study we subsequently investigated 65 index patients with autosomal dominant HSP, all of whom had previously been screened negative for SPAST mutations. Independent secondary samples, additional family members, and cDNA were analyzed to confirm positive findings. Results: Aberrant MLPA profiles were identified in 12 cases (18%). They exclusively affect SPAST, represent deletions, segregate with the disease, and are largely pedigree specific. Internal SPAST deletions entail expression of correspondingly shortened transcripts, which vary in stability. Age at onset in SPAST deletion carriers does not differ from that associated with other SPAST mutations. Conclusions: Partial SPAST deletions, but not SPAST amplifications and SPG3A copy number aberrations, represent an underestimated cause of autosomal dominant hereditary spastic paraplegia. Partial SPAST deletions are likely to act via haploinsufficiency.

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sought to derive a statement as to the usefulness of implementing copy number screening in routine diagnosis of patients with spastic paraplegia.

**Methods. Subjects.** Noncarriers in HSP pedigrees for which a causative mutation has been identified served as healthy controls. One DNA sample with a deletion spanning several SPAST exons and three DNA samples harboring different SPAST mutations within MLPA probe hybridization sequences were assembled from previous studies. To screen for as-yet-undetected copy number aberrations, 65 index patients with autosomal dominant HSP of Austrian and German origin were recruited by spastic paraplegia outpatient clinics and the German Network for Hereditary Movement Disorders. Diagnosis of autosomal dominant HSP based on the following diagnostic criteria: 1) pure spastic paraplegia or 2) spastic tetraparesis with earlier and more severe affection of lower limbs or 3) spastic paraplegia as an early and prominent sign of a degenerative disease affecting the nervous system and 4) positive family history of spastic gait disturbance with affected members in at least two generations and 5) other causes of the presenting symptoms excluded. All patients had been regarded SPAST mutation negative based on conventional exon-by-exon amplification followed by sequencing. When feasible, an independent second sample or samples from additional family members were collected upon identification of an aberrant MLPA profile in the index patient (see the table for details). Age at onset of symptoms was determined from clinical records.

mRNA was available from one of the control subjects and from affected individuals of three MLPA-positive families, including a patient known to carry the previously reported deletion of exons 13 to 16 of SPAST.

**Development of MLPA probe set targeting two autosomal dominant HSP genes.** Design, cloning, and purification of MLPA probes followed the general procedure outlined previously. The HSP MLPA probe mix contains 14 probes specific for all exons of SPG3A except exon 13, which consists of only 15 bp and is present only in an alternative transcript; for the isolated exon 1, two different probes are included. For SPAST, there are probes for all 17 exons with exon 1 targeted by two probes. In addition, 14 MLPA control probes specific for other human genes located on different chromosomes are included to guide copy number quantification. A complete list of all probes can be provided upon request.

**Detection of copy number aberrations.** Genomic DNA was prepared from peripheral leukocytes following standard procedures. For MLPA, 100 to 200 ng of DNA was heat denatured and incubated with the probe set for 16 hours at 60 °C. Hybridized probes were ligated and PCR-amplified with IRD800-labeled primers. PCR products were separated on an automated sequencer (LI-COR; Lincoln, NE). The intensity of each amplicon was measured densitometrically and exported to a Microsoft Excel spreadsheet. A relative signal value (RSV) for each SPG3A and SPAST amplification was derived by dividing its intensity by the sum of the intensities of the four nearest peaks representing probes for the other HSP gene or control MLPA probes. A normalized RSV (N-RSV) was generated by dividing each RSV by the corresponding mean from the six control DNA samples. As in previous MLPA studies, N-RSVs deviating by more than 25% from this mean were regarded aberrant.

**Analysis of transcripts.** mRNA was isolated from peripheral leukocytes using the PAX gene blood RNA system according to the manufacturer’s instructions (Qiagen, Hilden, Germany) and immediately reverse transcribed (iScript cDNA Synthesis Kit; Bio-Rad, Hercules, CA). Amplification of cDNA utilized exonic primers neighboring selected internal SPAST deletions; oligonucleotide sequences are available upon request.

**Results.** MLPA profiles in healthy control subjects and in individuals with known SPAST mutations. We first tested the probe mix on DNA from six healthy control individuals. This resulted in N-RSVs between 0.82 and 1.18 for all SPG3A- and SPAST-specific probes with 90% of values between 0.9 and 1.1 (figure 1). This high homogeneity qualified the use of these samples as a reference panel in subsequent analyses; it also made us confident in regarding values <0.75 or >1.25 as denoting an aberrant MLPA profile. We next analyzed a DNA sample harboring a deletion of exons 13 to 16 of SPAST. N-RSVs for the

<table>
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<th>Extent of deletion</th>
<th>Analysis of second DNA samples (s)</th>
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<tr>
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* See text for details.

MLPA = multiplex ligation-dependent probe amplification.

**Figure 1.** Control multiplex ligation-dependent probe amplification (MLPA) profiles. (A) Horizontal view of raw image obtained from separating MLPA products by gel electrophoresis on an automated sequencer. Product size increases from right to left. Each of the six lanes, corresponding to the analysis of six control samples, consists of 46 data points (14 SPG3A probes, 18 SPAST probes, and 14 control probes). (B) Normalized relative signal values (N-RSVs) of the six control DNAs. In general, the N-RSV for each probe is close to 1, indicating high homogeneity within these samples; deviation of >25% (indicated by dotted line) is restricted to one of the control MLPA probes (arrow).
affected exons were between 0.4 and 0.6, whereas all other values were in the normal range (figure 2A). We finally tested three DNAs containing mutations within MLPA probe hybridization sequences. Nonaberrant N-RSVs were obtained for a single base deletion (25-bp distance to MLPA probe ligation site) and a single base substitution (3-bp distance) (figure 2, B and C). In contrast, a 3-bp deletion (12-bp distance) generated a decreased N-RSV of 0.68 for the corresponding probe (figure 2D).

Confirmatory analyses. All aberrant MLPA profiles were confirmed in at least one rerun. The analysis of independently collected secondary samples and of DNA from additional family members confirmed the findings obtained on the primary sample and generally showed segregation with the disease phenotype. In one pedigree, however, deletion of SPAST exons 1 to 7 was found in an unaffected elderly individual but not in an 11-year-old child who shows several neurologic abnormalities including abnormal gait (see Discussion).

Amplification of SPAST cDNA from mutation carriers resulted in extra PCR products smaller than the expected size (figure 3). Sequencing revealed these products to lack the deleted exons (data not shown). Compared with transcript from the wild-type allele, the stability of the exon 5-to-7 deleted transcript, but not that of the exon 13-to-16 deletion, appeared strongly reduced (figure 3, A, C); no statement as to relative transcript stability was possible for the exon 5-to-15 deletion owing to failure of amplification of the wild-type cDNA sequence (expected size 1.7 kb; figure 3B). The table indicates the level of confirmation for each aberrant MLPA profile.

Discussion. The current study introduces an MLPA-based tool for the detection of genomic copy number aberrations in the HSP genes SPG3A and SPAST. This tool was validated by showing homogeneous profiles across control samples (figure 1) and specific signal reduction associated with a known multiexonic SPAST deletion (figure 2A). Consistent with previously reported findings,17 two single-nucleotide mutations, namely, a substitution 3 bp from the MLPA probe ligation site and a single-base substitution (3-bp distance) (figure 2B and C). In contrast, a 3-bp deletion (12-bp distance) generated a decreased N-RSV of 0.68 for the corresponding probe (figure 2D)

MLPA profiles in HSP patients previously screened negative for SPAST mutations. Sixty-five index patients, all of whom had previously been screened negative for mutations in SPAST by sequencing of the entire gene, were analyzed by MLPA. Aberrant profiles were identified in 12 cases (18%; table). Only MLPA probes specific to SPAST contributed to the aberrance of the peak patterns, and in all instances signals were reduced rather than increased (figure 2E). In general, multiple probes localizing to adjacent exons or targeting different regions within one exon were affected. In the two cases where only the value of a single probe was decreased (exon 10, exon 17; table), resequencing confirmed that no alteration within the probe hybridization sequence was present (data not shown). With the exception of the exon 5-to-15 deletion, which was found in an Austrian as well as in a German family, all deletions identified were unique (table). Mean age at onset of affected individuals from SPAST deletion families was 30.6 ± 16.1 years and varied widely (range 1 to 60 years; table).

To date, only two large genomic aberrations had been identified in patients with autosomal dominant HSP16; both are deletions, affect SPAST, and, due to having been missed by conventional mutation screening, were uncovered only through labor-intensive cDNA analysis or Southern hybridization.
Using MLPA, we identified an additional 12 cases of partial \( \text{SPAST} \) deletions (figure 2E; table). By investigation of affected and unaffected family members, complete congruence between presence of the deletion and disease status could be shown for all but one pedigree. In this family, the deletion in the index patient was also found in an unaffected elderly relative who, on the other hand, represents a candidate asymptomatic carrier according to pedigree structure. More noticeable was the absence of the deletion in a younger family member with neurologic abnormalities. According to her parents, this girl had an abnormal gait from age 3 on. At examination at age 10, the girl walked with genua valga and inward rotation of the right foot. There was mild ataxia in tandem walking. Although the patellar and Achilles tendon reflexes were very brisk, there was no spastic muscle tone in the legs, no gait spasticity, and a negative Babinski sign. Causes other than HSP need therefore be considered, and further efforts to clarify the role of the deletion in this family are under way.

Our identification of 12 partial \( \text{SPAST} \) deletions was based on a cohort of 65 patients previously screened negative for \( \text{SPAST} \) mutations by conventional sequencing approaches. With such a cohort representing 60% of autosomal dominant HSP cases, the frequency of partial \( \text{SPAST} \) deletions in this disease is estimated to be approximately 11% (0.6 x 12/65). This number is comparable with that of other classes of \( \text{SPAST} \) alterations (i.e., missense, splice site, nonsense, and frameshift mutations) and totals the overall involvement of \( \text{SPAST} \) in autosomal dominant HSP to >50%. In light of these data, it is likely that a large proportion of the pedigrees showing so-far-unexplained linkage to \( \text{SPG3A} \) harbors large deletions rather than alterations in intronic/regulatory sequences or in an occasionally presumed nearby second HSP locus.

The partial \( \text{SPAST} \) deletions identified here are likely to be pathogenic through haploinsufficiency as they should either 1) impede translation (e.g., exon 1-to-3 deletion), 2) create a preterminal stop codon thereby making the transcript a likely substrate for nonsense-mediated decay even though the remaining exons may be properly spliced (e.g., exon 5-to-7 deletion; figure 3A), or 3) remove large parts of the functionally important AAA domain (e.g., exon 5-to-15 deletion). A similar interference with expression of functional protein would be expected to result from partial duplications of \( \text{SPAST} \). Several cases for which both deletion and duplication of a specific genomic segment occur and are associated with inherited diseases have been described and are interpreted as representing reciprocal recombination events between repetitive sequences. Despite the relatively high density of \( \text{Alu} \) elements in \( \text{SPAST} \) (36.6% compared with the average 30.4%), lack of a partial \( \text{SPAST} \) duplication in our cohort and apparent nonrecurrence of the deletions identified suggests recombination-independent mechanisms to prevail in the deletion-positive cases. Detailed analysis of the breakpoints is needed to clarify this issue.

In contrast to \( \text{SPAST} \), no copy number aberrations affecting \( \text{SPG3A} \) were identified during this screen. This is consistent with the spectrum of mutations reported previously in both genes: Whereas alterations in \( \text{SPG3A} \) comprise almost exclusively missense mutations, nonsense, frameshift, as well as splice site mutations, all presumably acting via haploinsufficiency, account for a large proportion of \( \text{SPAST} \) mutations. Another not mutually exclusive but more mechanistic explanation for failure to identify \( \text{SPG3A} \) copy number aberrations is the extremely low density of only 14.4% of \( \text{Alu} \) elements in this gene.

Mutational class in \( \text{SPAST} \) could, so far, not be correlated with clinical variables. Similarly, partial \( \text{SPAST} \) deletions do not seem to be different from other mutations as regards age at onset as the main quantifiable clinical variable (mean 30.6 ± 16.1 vs 28.6 in a meta-analysis on previously reported mutations). Although we cannot exclude that larger sample sizes or inclusion of additional clinical measurements may reveal significant differences, this may be indicative of a common molecular pathomechanism underlying all \( \text{SPAST} \) mutations.

That the majority of the \( \text{SPAST} \) deletions are unique for each pedigree hampers development of a PCR-based detection assay similar to those applied in other inherited diseases frequently caused by
genomic deletions. With several cases involving the transcription start site at the 5′ end of the gene (table) or conferring instability to the transcript from the mutated allele (figure 3A), more generalized alternative screens such as cDNA analysis and the protein truncation test are likely to also fail in detecting many of the SPAST deletions presented here. Similarly, not knowing the extent of the aberration renders Southern hybridization a potentially inefficient screening method. MPLA-based mutation screening may therefore be considered in the routine diagnosis of HSP patients.

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References


