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**220 patients with autosomal dominant spastic paraplegia do not display mutations in the *SLC33A1*-gene (SPG42)**

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**References**

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**Abstract**

The most frequent causes of autosomal dominant HSP (ADHSP) are mutations in the *SPAST*-gene (SPG4 locus). However, roughly 60% of the patients are negative for *SPAST*-mutations despite their family history is compatible with autosomal dominant inheritance. A mutation in the gene for an acetyl-CoA transporter (*SLC33A1*) has recently been reported in one Chinese family to cause ADHSP type SPG42. Here we screened 220 independent *SPAST*-mutation-negative ADHSP samples for mutations in the *SLC33A1*-gene by high resolution melting curve analysis (HRM). Conspicuous samples were validated by direct sequencing. Moreover, copy number variations affecting *SLC33A1* were screened by multiplex ligation-dependent probe amplification assay (MLPA). We could not identify potentially disease-causing mutations in our patients either by mutation scanning or by gene dosage analysis, for the latter specific positive controls are not available to date. As our sample represents ADHSP patients for which *SPAST*-mutations and almost in all cases *ATLI*- and *REEP1*-mutations had been excluded, we consider *SLC33A1*-gene mutations as very rare in a European ADHSP cohort if present at all. To date, as SPG42 still lacks to be identified in a second, unrelated family, systematic genetic testing for *SLC33A1*-mutations is not recommended.

## Introduction

The hereditary spastic paraplegias (HSPs) are a group of neurodegenerative diseases clinically characterized by progressive lower limb spasticity, pyramidal weakness and extensor plantar responses. The main neuropathological feature of these conditions is declining degeneration of the corticospinal tracts. Pure (uncomplicated) and complicated forms of HSP have been described according to the presence of additional neurological features like cerebellar ataxia, peripheral neuropathy and/or cognitive impairment in addition to spastic paraparesis. In addition to clinical manifestation, age at onset and genetic background are also highly heterogeneous.<sup>1,2</sup> To date, at least 46 different loci have been mapped, associated with autosomal dominant (AD), autosomal recessive (AR), and X-linked (XL) mode of inheritance.<sup>1-3</sup> 17 responsible genes have been identified according to the HUGO and OMIM databases. Consistent with this high variability, HSP gene products seem to be involved in a wide range of cellular functions and pathogenic mechanisms.<sup>4</sup> Autosomal dominant inheritance is the most common trait in pure HSP. At present 18 loci have been linked to ADHSP, and 9 of the genes have been identified. Mutations in the genes *SPASTIN* (MIM# 604277; SPG4, MIM# 182601), *ATL1* (MIM# 606439; SPG3, MIM# 182600), *KIF5A* (MIM# 602821; SPG10, MIM# 604187) and *REEP1* (MIM# 609139; SPG31, MIM# 610250) account for ~ 40%, ~10%, ~3% and ~8% of all autosomal dominant HSP patients, respectively.<sup>2,5-7</sup> The remaining HSP genes appear to be relatively rare.<sup>8,9</sup> Spastic paraplegia type 42 (SPG42, MIM# 612539) was recently mapped to the 3q24-q26 chromosomal region in a single Chinese family

presenting with pure ADHSP. Based on the detection of a serine to arginine substitution at the codon113 in this family, the authors reported *SLC33A1* (MIM# 603690) to be the responsible gene.<sup>10</sup> The protein product of *SLC33A1* is an acetyl-CoA transporter, which serves as a substrate of acetyltransferases that modify the sialyl residues of gangliosides and glycoproteins.<sup>11</sup> It is supposed that the modification of gangliosides and glycoproteins by acetylation probably plays a critical role in outgrowth and maintenance of axons of motor neurons.<sup>10</sup>

The frequency of SPG42 and its associated phenotypes is not known since no further families with *SLC33A1* mutations have been described so far.<sup>10</sup> The purpose of the present study was to determine the frequency of SPG42 by screening 220 European (German, French, Norwegian) ADHSP patients for conventional and gene dosage mutations.

## **Patients and Methods**

### **Patients**

A total of 220 DNA samples of unrelated HSP patients were recruited via the European Spastic Paraplegia network (EUROSPA) and the Norwegian HSP outpatient clinic. All patients were assessed by experienced neurologists. For all patients a family history consistent with dominant inheritance was reported. All index patients were of European descent (Germany (n=45), France (n=147) and Norwegian (n=28)). Criteria for inclusion in the study were mutation-negative for *SPAST*. Mutations in the *ATL1*- und *REEP1*-gene had previously been excluded

by direct sequencing and multiplex ligation-dependent probe amplification (MLPA) at the following frequencies: SPG3A sequencing in 55% of cases, SPG3A MLPA in 85% and SPG31 (sequencing and MLPA) in 84% of cases.<sup>7,9,12-</sup>

<sup>14</sup> The HSP phenotype was pure in 125 (57%) and complicated in 95 (43%) patients. Informed consent was obtained in all cases.

## Methods

### Analysis of the *SLC33A1*-gene

DNA was extracted from peripheral blood samples following standard protocols. The 6 coding exons of the *SLC33A1*-gene (ENSG00000169359, ENST00000359479) were screened by high resolution melting curve analysis (HRM) in all patients. The primers were designed to flank the coding regions and to amplify fragments of an average size of 250 bp (with the largest amplicon spanning 298 bp). PCR and HRM were performed in a single run on a LightCycler<sup>®</sup>480 instrument (Roche Diagnostics, Mannheim, Germany). A normal control DNA, which had been completely sequenced, served as the HRM baseline sample in each fragment. Samples with an aberrant melting profile were assigned for validation sequencing. These samples were re-amplified from genomic DNA and directly sequenced in order to identify or exclude sequence variants. Forward and reverse sequence reactions were performed with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems Inc, Foster City, CA, USA) using the same primers. The sequence products were analyzed on an ABI3100 Genetic Analyzer (Applied Biosystems Inc, Foster City, CA, USA). See supplement for detailed experimental procedures and primer sequences (supplementary table 1).

In order to screen for copy number aberrations affecting *SLC33A1* we designed a multiplex ligation-dependent probe amplification (MLPA) assay. It targets the coding sequence of each exon as well as the promoter sequence of *SLC33A1* gene



with one probe each. Three probes localising to different chromosomes are included as references (probe sequences are available upon request). Pertinent synthetic oligonucleotides (MWG Biotech, Ebersberg, Germany) and reagents from EK1 kit provided by MRC-Holland (Amsterdam, The Netherlands) were used. MLPA reactions were performed according to the instructions of the manufacturers. Analysis of MLPA data was carried out as described previously.<sup>15</sup>

## Results and Discussion

HRM analysis detected an aberrant melting profile caused by an A>G substitution at position 512 of the coding sequence which causes a p.D171G substitution in 16 index patients (Figure 1). This sequence abnormality represents the known single nucleotide polymorphism (SNP) rs3804769 according to the SNP database.<sup>16</sup> No other sequence alterations were detected. Since HRM analysis is a screening method with ~99% sensitivity<sup>17,18</sup> we handled the results with care. Along with this high sensitivity, HRM is a simple, rapid and low cost method to screen for unknown sequence variants in a high-throughput modality. Compared to other methods, like denaturing high-performance liquid chromatography (dHPLC), HRM offers at least two additional benefits: (1) the temperature gradient covers all potential melting domains of amplicons, which provides superior detection sensitivity. (2) the closed-tube method reduces post-PCR manipulations and the risk of pipetting errors and contaminations.<sup>17,18</sup> Moreover, our identification of the rs3804769 SNP represents a positive control and argues for the general validity of our approach.

Similar to all PCR-based techniques, large insertions or deletions are usually not detected during gene scanning by HRM.<sup>17</sup> This mutational class is frequent (approx. 20%) in SPG4 and is also relevant for SPG31.<sup>7,14,19</sup> Both forms are associated with pure autosomal dominant HSP. To assess quantitative changes in *SLC33A1* copy number, MLPA was used but no rearrangements were identified. Since no duplications or deletions of *SLC33A1* have been reported so far, no positive control was available. However, the technique can be regarded as rather

robust and homemade probe sets developed by other groups (i.e. Ganesamoorthy *et al.*, 2009)<sup>20</sup> as well as our groups (unpublished) have been successfully used in unambiguously detecting copy number alterations. Moreover, high overall data quality (data not shown) argues for the applicability of this assay, therefore we believe our findings are likely not false negative.

Screening for gene dosage seemed especially important in SPG42 since haploinsufficiency is supposed to be the pathogenic disease mechanism.<sup>10</sup>

Additionally, *SLC33A1* has an unusual high content of Alu sequences (38.1% compared to the ~10% genomic average<sup>21</sup>). In other genes such high values are associated with an increased susceptibility to genomic deletions (i.e. *MSH2*).<sup>22</sup> Still, MLPA failed to identify this potential pathological mutation class in our study.

The 220 index patients screened in the present study represent a cohort of more than 500 consecutive ADHSP index patients as mutations in common HSP genes (*SPASTIN*, *ATL1* and *REEPI*), which account for >50% of our ADHSP diagnoses, had already been excluded. Based upon data published previously by Lin *et al* (2008)<sup>10</sup> the results of this study can be interpreted in two ways. First, the lack of *SLC33A1* mutations in our sample may indicate that SPG42 is an extremely rare form of ADHSP at least in our European cohort. An alternative explanation for the negative finding of our study may consider the *SLC33A1*-gene to not be the true disease gene for the SPG42 HSP subtype. As only a single SPG42 family has been described so far, it is difficult to draw any genotype/phenotype correlations. In this Chinese family 20 affected subjects presented with a “pure” form of HSP

starting between 4 and 42 years of age, only some had wasting of lower limbs mentioned apart from pyramidal symptoms.

Identification of additional SPG42 families would help to decide whether SPG42 represents a pure spastic paraplegia or whether a complicated course might be seen in other patients with different mutations. Moreover, a next generation sequencing approach encompassing the whole locus in the only SPG42 family known so far should help to search for sequence alterations in the about 125 genes, which haven't been screened yet.

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### **Conflict of interest**

The authors declare no conflict of interest.

Supplementary information is available at ESHG's website.

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### **Web resources**

The URLs for data presented herein are as follows:

HUGO Gene Nomenclature Committee. <http://www.genenames.org>

Online Medelian Inheritance in Man (OMIM),

<http://www.ncbi.nlm.nih.gov/omim/>

UCSC Genome Browser, <http://genome.ucsc.edu>

Figure 1: Known SNP rs3804769 in *SLC33A1*

In total, 16 patients were found to be heterozygous for rs3804769 in *SLC33A1* Exon 1 (c.512A>G, p.D171G). 16 variant alleles (7.0%) were present in 440 sample chromosomes, as assessed by high resolution melting curve analysis (left part). Difference plot analysis revealed the heterozygous group (red) in addition to the homozygous samples (blue). Heterozygosity has been validated by conventional sequencing (right part).

Normalized and Temp-Shifted Difference Plot

