

Original Article

Whole exome sequencing identifies a novel *SPG4* mutation in a Chinese family with hereditary spastic paraplegias

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Abstract: Hereditary spastic paraplegias (HSPs) are a group of neurodegenerative disorders of highly clinical and genetic heterogeneity. *SPG4* (*SPAST*) is the most common type of pure autosomal dominant hereditary spastic paraplegia (ADHSP) and its mutations account for 40% of all mutations associated with the ADHSP. The purpose of this study was to identify the causative gene mutation and explore the genotype-phenotype correlation through the investigation of a Chinese Han family with ADHSP, which could lay solid foundation for further study of the pathogenesis and provide the basis for prenatal diagnosis for this disease. Whole exome sequencing (WES) was performed on subjects with HSP from a Chinese family in Shandong Province. Systematic clinical analysis and 3.0T Magnetic resonance imaging (MRI) scans of brain and spinal cord on the affected proband were also accomplished. As a result, a novel nonsense *SPG4* mutation, a single-nucleotide change from C to T which caused a substitution from glutamine to an immature stop codon at codon 536 (c.1606C>T, p.Gln536X), was identified. This mutation co-segregates with the HSP patients in this pedigree. MRI revealed no significant atrophy of spinal cord and brain in the proband. In conclusion, our finding suggests that the novel nonsense mutation in *SPG4* is causative to HSP and it's of great significance in supplementing the mutational spectrum of the *SPG4* and explaining the mechanism of HSPs. Our study also indicates that WES can be an efficient and rapid diagnostic tool for some complex and genetically heterogeneous diseases.

Keywords: Hereditary spastic paraplegias, *SPG4* gene, whole exome sequencing, magnetic resonance imaging

Introduction

Hereditary spastic paraplegias (HSPs) are a group of neurodegenerative disorders with clinical and genetic heterogeneity characterized by progressive muscular tension of bilateral lower limbs, muscle weakness and spasticity that featured predominantly, but not exclusively [1]. In general, upper limbs are rarely affected and if involved, spasticity of lower limbs usually appears before it by several years [2]. Clinically, HSPs are generally divided into pure and complicated forms and people with complex HSP may exhibit additional neurologic or systemic abnormalities such as mental and cognitive changes, optic atrophy, amyotrophy, ataxia, de-

afness, ichthyosis, and/or peripheral neuropathy [3]. Spastic paraplegia associated symptoms may firstly appear at any age even in same subtype and progress continuously over several years with no significantly shortening life expectancy (at least it's true for pure forms of this disease) [4]. Moreover, disease severity, degree of disability and rate of progression for HSP patients can differ between families and even intra families.

In general, HSPs are transmitted according to autosomal dominant (AD) which accounts for the maximum proportion (70-80%) [5], autosomal recessive (AR), X-linked recessive (XR) and maternal inheritance forms [6]. The candidate

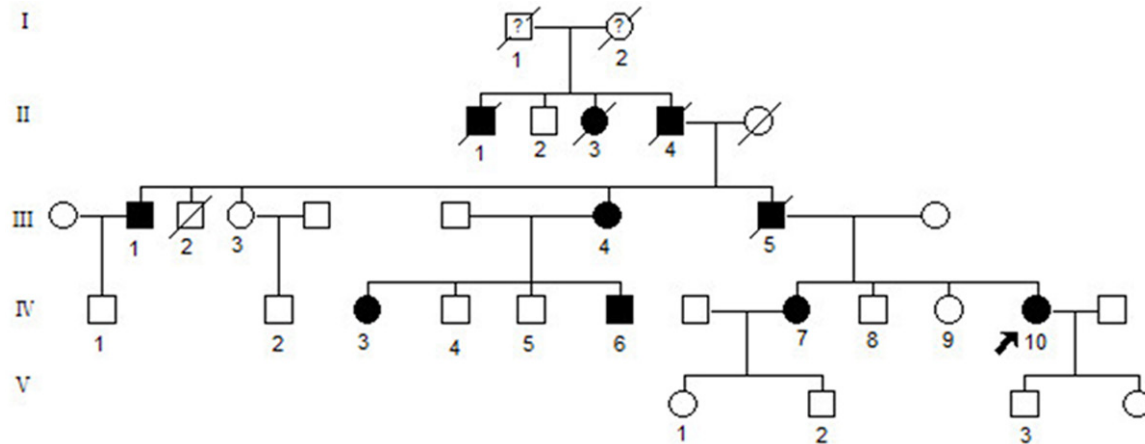


Figure 1. Pedigree of the family with hereditary spastic paraplegia. Squares and circles indicate males and females, respectively. Darkened symbols represent affected members. The black arrow indicates the index case IV10.

genes associated with ADHSP mainly include *ATL1* (*SPG3*), *SPAST* (*SPG4*), *NIPA1* (*SPG6*), *KIAA0196* (*SPG8*), *KIF5A* (*SPG10*), *RNT2* (*SPG12*), *SPGD1* (*SPG13*), *BSCL2* (*SPG17*), *REEP1* (*SPG31*) and so on [7]. Among them, *SPG4* is the most common type of pure ADHSP (40% of the familial and 6-15% of the sporadic cases), and *SPG3A* is the second most common (10%) [8], respectively. *SPG4*, also known as *SPAST*, is located on 2p24-p21 and encodes SPASTIN, a member of the AAA (ATPase associated with various cellular activities) family of proteins. At least twelve causative genes have been confirmed to be related to ARHSP such as *CYP7B1* (*SPG5*), *SPG7* (*SPG7*) [7] and so on. Up till now, due to technological revolution in molecular genetics, at least 76 different spastic gait disease-loci with 59 corresponding spastic paraplegia genes have been identified [9], which have brought great difficulties to traditional genetic diagnosis for HSP.

Although the conventional technical examinations including a magnetic resonance imaging (MRI) of brain and spinal cord, a normal lumbar puncture and an electrophysiological examination [10] except clinical features may be useful for the diagnosis for HSP, we are unable to differentiate the various HSP subtypes according to clinical parameters simply because of its clinical and genetic heterogeneity [11]. With the development of the next generation sequencing (NGS) technologies, whole exome sequencing (WES) has been proven to be a practical and powerful method for identifying causative mutations in various Mendelian disorders [12],

including rare diseases with genetic heterogeneity, such as HSP.

In this study, clinical and neuroradiological features of a Chinese family with ADHSP were described in detail. Furthermore, we designed to integrate an approach of WES with Sanger sequencing validation to identify the disease-causing mutation for the proband.

Patients and methods

Patients

This study was approved by the ethics committee of the Affiliated Hospital of Qingdao University. The subjects were from a Chinese Han family in Shandong province. A 50-year-old female proband (IV10, **Figure 1**) in the family was clinically diagnosed with pure ADHSP in neurology department in the Affiliated Hospital of Qingdao University (Shandong, China) according to Harding's criteria [13]. Her father and her elder sister were also troubled with HSP (**Figure 1**). She experienced a series of detailed neurological and MRI examinations. After obtained written informed consent, blood samples were collected from the proband (IV10, **Figure 1**), her elder affected sister (IV7, **Figure 1**) and a normal individual (IV9, **Figure 1**) in this family.

Whole exome sequencing

We extracted DNA samples from 200 µl peripheral venous blood using a Qiagen DNA extraction kit (Qiagen, Hilden, Germany). WES was

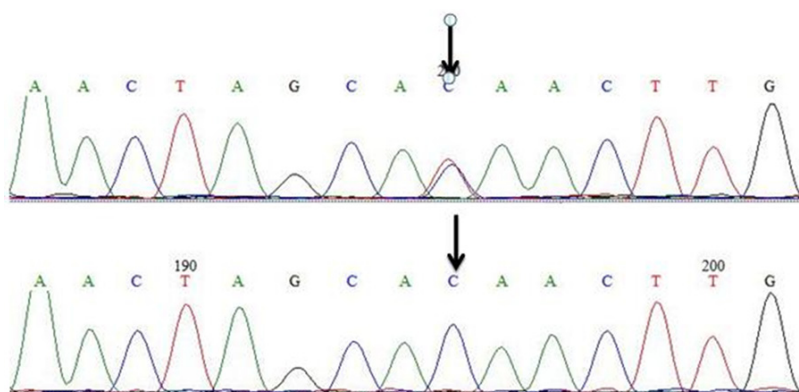


Figure 2. Sequence chromatograms. The female carried the novel mutation, c.1606C>T (p.Gln536X). The black arrow indicates the mutational loci which leads to the replacement of glutamine (Gln) with a premature termination codon at codon 536. The healthy control is shown on the below.

carried out on the proband (IV10, **Figure 1**) and her affected elder sister (IV7, **Figure 1**) in the family with the use of human exome capture which was performed according to the protocol from Illumina's TruSeq Exome Enrichment Guide (SureSelectXT Target Enrichment System for Illumina Paired-End Sequencing Library, Agilent). The Agilent Human All Exon 50 Mb Exome Enrichment kit was selected as exome enrichment probe sets. The Genomic DNA libraries were subjected to the manufacturer's instructions (Illumina, San Diego, CA, USA). In brief, 5 ug genomic DNA was mixed in 80 ul EB buffer and fragmented in a Bioruptor (Diagenode) to 100-500 bp fragments. We performed gel extraction to recycled DNA fragments between 150-250 bp, then end repair and size selection procedure were performed by T4 DNA poly and Klenow poly cleave 3'. An 'A' base was added to the 3' end using Klenow 3' to 5' exo minus, then DNA fragments were ligated to the Illumina multi-PE-adaptor. Subsequently, PCR amplification of 12 cycles was carried out of the DNA product, the reaction system was consisted of 1 ml of Illumina multi-PE primer #1 (25 mM), 1 ml of Illumina multi-PE primer #2 (0.5 mM), and 1 ml of Illumina index primer (25 mM).

Captured DNA libraries sequencing was executed with Illumina HiSeq 2000 platform, which yielded 200 (2*100) bp from the final library fragments using V2 reagent 1.8 software (Illumina; data after 22nd June, 2011) was used to perform base calling. The obtained sequence reads were in accordance with the

human genome reference sequence (NCBI36/hg18), and variations were identified using the software tool supplied with the instrument. Finally we got 62.09 M high quality reads, and 44.85 M were mapped to the reference genome, the mean depth of the target region was 114.836. Targeted bases with at least 50 × was 75.81%, 20 × 82.23%, 10 × 89.04%, 4 × 93.56%, 1 × 96.09%. Further analysis was performed based on these general statistics.

All identified *SPG4* variations were annotated with information to identify candidate mutations displaying the depth of coverage, conservation across species, percentage of reads with the variant, novelty, potential splice site alteration, and likelihood that a variation is deleterious to the protein. This information was extracted from reference data sets or computed in bulk for all variations.

Sanger sequencing validation

The *SPG4* variant of the proband and her elder sister identified by WES was validated using Sanger sequencing. Two fragments covering the coding sequence of 5'-TAGCACAACCTGCTAGGT-3' were amplified using *SPG4* primer pairs for exon 14 (Forward: 5'-TTGCTTAACCCAGGATA-3' and Reverse: 5'-CAAAGGAGGTAGAGGATG-3'). We used identical amplification conditions for both primer pairs in a total volume of 25 ul containing 250 nM dNTPs, 100 ng of template DNA, 0.5 mM of each primer, and 1.25 U AmpliTaq Gold DNA polymerase in 1 × reaction buffer (10 mM Tris HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl₂). PCR amplifications were performed as the following steps that with an initial denaturing step at 94°C for 5 min, then 35 cycles of: 94°C for 30 s, 58°C for 60 s, 72°C for 30 s, followed by 10 min of final extension at 72°C. Amplified PCR products were purified and sequenced using the appropriate PCR primers and the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and run on an automated sequencer, ABI 3730XL

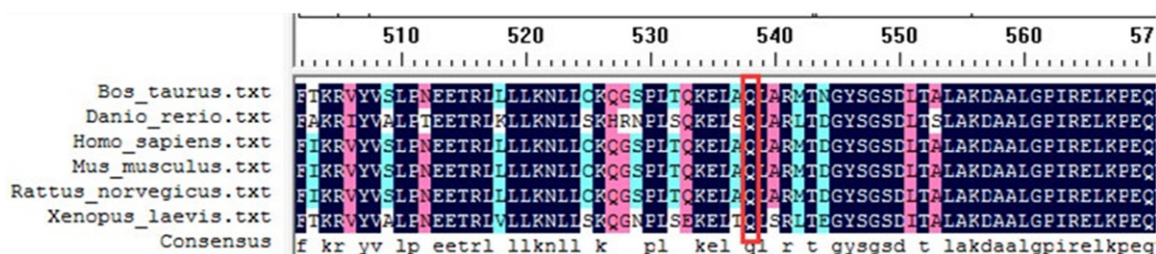


Figure 3. Multiple-sequence alignments. Multiple-sequence alignments of the SPG4 protein including Bos taurus, Danio rerio, Mus musculus, Rattus norvegicus, Xenopus laevis and Homo sapiens. The p.Gln536X variant is located within a highly conserved region as shown in the red rectangle.

(Applied Biosystems) to perform mutational analysis.

Magnetic resonance imaging

MRI of spinal cord (including cervical and thoracic spine) and brain was performed in 3.0-Tesla clinical scanner (GE HDx 3.0T MR System, GE Medical System, Milwaukee, USA) for the affected individual (IV10, **Figure 1**). The sequences of MRI were sagittal FSE T1WI, FSE T2WI, fat-saturation FSE T2WI, axial FSE T2WI for spine, and sagittal FSE T1WI, axial FSE T1WI, FSE T2WI, FLAIR, DWI for brain respectively. Fifty healthy age- and sex-matched individuals had been examined previously using the same MRI techniques with the same clinical scanner in the same hospital for comparison with the affected female.

Results

Clinical phenotype

The affected individual was diagnosed with pure ADHSP without cognitive impairment and other psychological symptoms. The onset of her spastic gait problem manifested as less expressed stumble, stiffness and weakness in lower limbs occurred when she was 12 and had slowly progressed. Pes cavus was entirely found in her daily life. Following this, she had difficulties in running and walking without help at the age of 30. When she was 45 years old, she became a wheelchair-bound. In our clinical department, neurological examination revealed weakness and spasticity in lower limbs, positive hyperreflexia, bilateral Babinski and Chaddock signs. The patient complained of sensory impairment for at least six years. The upper limbs were also involved in tendon hyperreflexia, grip strength and muscular tone acted normally.

Genetic analysis

WES and several filtering steps were occupied to exclude nongenetic variants by filtering the database of dbSNP and 1000 genomes to select for nucleotide changes predicted to play a damaging effect on the SPASTIN by SIFT (Sorting intolerant from tolerant) and PolyPhen-2 (Polymorphism Phenotyping v2). The depth of coverage for c.1606C>T in exon 14 is 75 × and it indicated high reliability of sequencing. Sanger sequencing confirmation of the proband and her elder sister revealed a compound heterozygous genotype, and it predicted to change a glutamine to a stop codon at codon 536 in exon 14 based on the novel nonsense variant (c.1606C>T, p.Gln536X) (**Figure 2**). According to the HSP mutation database (<https://reseq.biosciencedbc.jp/resequence/GeneDetail.do?targetId=9&genId=EG6683>), the mutation (c.1606C>T) has never been previously demonstrated. Co-segregation analysis of this pedigree revealed that the proband (IV10, **Figure 1**) and her elder affected sister (IV7, **Figure 1**) carried the same nonsense mutation while her normal sister (IV9, **Figure 1**) didn't carry it. Additional genetic studies of the family history revealed that this mutation was inherited from her father and it was not observed in the 100 control individuals.

Bioinformatic analysis of SPG4 mutation

The SPG4 protein sequences of various animal species including Mus musculus, Rattus norvegicus, Bos taurus, Danio rerio, Xenopus laevis and Homo sapiens were obtained from NCBI and UCSC websites and multiple-sequence alignments were performed by using Vector NTI software. The p.Gln536X variant was found to be located in a highly conserved region of the SPG4 protein (**Figure 3**).

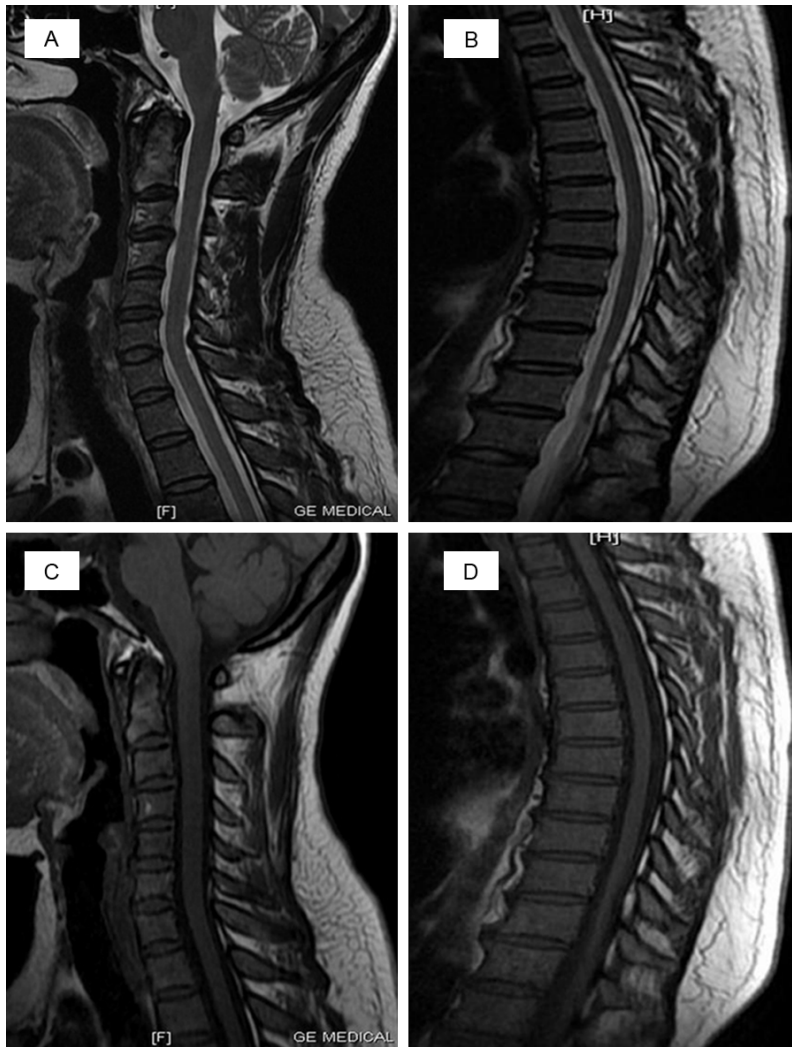


Figure 4. MRI of the proband. MRI shows no clear abnormality in the cervical and thoracic spinal cord of the patient with *SPG4*. There was no signal abnormal signal intensity on sagittal FSE T2WI and SE T1WI of cervical (A and C) and thoracic spinal cord (B and D).

MRI analysis

No significant differences were found in signal intensities and shape on different sequences, axial diameter and area of spinal cord from the age- and gender-matched controls in the cervical or thoracic segments and brain in this *SPG4*-HSP patient through MRI scans (**Figure 4**).

Discussion

In this study, we identify a causative gene mutation which affects RNA transcripts and causes a premature stop codon in exon 14 in *SPG4* through the investigation of a Chinese Han family with ADHSP by WES.

HSP is generally regarded to be a rare neurodegenerative disorder characterized pathologically by degeneration of the cortico-spinal tract motor neurons and results in weakness and progressive spasticity of the lower limbs. Although the etiology and pathogenesis of HSP remains unclear, mutations in *SPG4* which contains 17 exons are responsible for almost 50% of pure ADHSP patients [14]. The SPASTIN, a microtubule-severing protein encoded by *SPG4* has been studied to be implicated in the remodeling of protein complexes by ATP hydrolysis and the coordination of axonal microtubule interactions with the tubular endoplasmic reticulum network [15, 16]. Despite that SPASTIN activity was decreased differently by different *SPG4* mutations, clear correlation between the mutational type and the severity of the phenotype remains unidentified [17, 18]. Since the identification of *SPG4* as a causative gene for HSP, to date, over 200 different *SPG4* mutations including missense, nonsense, splice site, insertions, small and large deletions have been found [17, 19].

Most ADHSP-*SPG4* cases are diagnosed typical 'pure' or 'uncomplicated' subtype with gait impairment on account of weakness and spasticity of the lower extremities (each of variable degree and age of onset) but with no functional loss in upper limbs or shorter lifespan [20]. Occasionally, pure ADHSP-*SPG4* is along with sensory disturbances or bladder dysfunction [21]. In our study, the proband in this pedigree was disturbed by mild sensory abnormalities of the lower limbs (reduced vibration sense), pes cavus, and tendon hyperreflexia but without other features such as bladder involvement, mental and cognitive changes, peripheral neu-

ropathy and other symptoms. The clinical manifestations indicated that the woman had similar characteristics to most patients in previous studies of pure ADHSP. However, in the relatively few cases as well as the proband we checked, weakness and hyperreflexia in both upper limbs are atypical symptoms and cranial nerves are rarely involved in HSP [22]. Although *SPG4* is generally reported to be a late-onset HSP, the onset age may vary between 1 and 63 years old and peak at 10 and 30 years old [7]. Similarly, the woman in our study developed the associated symptoms such as stumble, stiffness and weakness firstly in his teens. Clinical heterogeneity mainly reflected in that mutations in a same region in *SPG4* would result in different phenotypes. Currently, there is still not any explicit explanation for the genotype-phenotype correlation.

Furthermore, *SPG4* has a relatively less prominent and severe atrophy of spinal cord than *SPG6*, *SPG8* and no apparent connection between the degree of spinal cord atrophy and the severity of the disease has been demonstrated in any genetic subtype of HSP [23]. In this study, MRI of spinal cord and brain also showed no noticeable pathological changes compared with the age- and sex-matched individuals.

The conventional diagnostic method of spastic paraplegia is differential diagnoses with other diseases such as cerebral palsy, multiple sclerosis, motor neuron disease and so on. Age and nature of onset, slow and subtle progression of symptoms, presence of a family history, and other clinical features are all the valuable clues [24]. In sporadic cases, because of the absence of a family history, the confirmed diagnosis of HSP depends on exclusion. Currently, many patients with HSP and other movement disorders remain difficult to diagnose by reasons of both clinical and genetic heterogeneity, unknown of the pathogenesis, and the ever growing number of new loci and causative genes [25]. HSP makes a tremendous difference in the quality of life and may develop progressively to be the ultimate paralysis, however, there is no efficient therapeutic method for it [5]. Consequently, gene diagnoses and prenatal genetic detection is much more important. It is even much more difficult to determine which gene is most likely associated with the affected indi-

viduals based on only clinical manifestations. In addition, the conventional molecular diagnoses were usually both time-consuming and expensive. With the progress of technology, WES is becoming a standard tool to discover genes underlying rare monogenic diseases and provide important guidance for finding rare variants influencing risk of common diseases [12]. According to a WES study of 250 probands, the overall rate of a positive molecular diagnosis was 25%, which is markedly higher than previously reported by other traditional genetics methods such as karyotype analysis [26, 27]. Immense amounts of studies have demonstrated that only a small number of affected family members or affected unrelated individuals are needed when WES is performed and it is really a powerful, efficient, and cost-effective strategy for diagnosing rare monogenic disorders. Since the first successful application of exome sequencing to discover the mutated gene for Miller syndrome [12], it was widely used in genetic diseases, such as axonal Charcot-Marie-Tooth disease [28]. Caroli Disease [29]. Non-syndromic autosomal recessive Retinitis Pigmentosa (arRP) [30] and so on.

In conclusion, we combined WES with Sanger sequencing to report a novel nonsense mutation of *SPG4* associated with HSP in a Chinese family. All the examinations and analysis indicated that this novel mutation is associated with HSP. Moreover, the WES can be a credible and direct diagnostic tool for identifying some complex and genetically heterogeneous diseases. However, further study of the pathogenic mechanisms of HSP and practical treatment plans still call for our deep investigation.

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Disclosure of conflict of interest

None.

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