

## Polymorphism in Spg4 as an Underlying Cause of Hereditary Spastic Paraplegia

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**Abstract: Background:** This study was done to find out the genetic cause of hereditary spastic paraplegia and determine the existing polymorphism. This can lead to opening of a new horizon in the field of hereditary spastic paraplegia. Study may provide basis for genetic counseling of these patients.

**Objective of study:** The objective of the study is to find out new or existing gene which can cause hereditary spastic paraplegia. Find out the polymorphism in this gene which can lead to hereditary spastic paraplegia.

**Method:** The present study was undertaken at Department of Anatomy, Institute of Medical Science, Sir Sunderlal Hospital, Banaras Hindu University, Varanasi, Uttar Pradesh, India. It was a cross sectional hospital based study over a period of 2 years. Ethical approval was obtained for study from institute. A written informed consent was obtained from the patients and family member. A pedigree was drawn of three generation. Blood sample of 5 ml was collected from the patient and family members. DNA was isolated from the blood samples. DNA of clients with hereditary spastic paraplegia were considered as experimental sample and DNA of family member without phenotype was consider as control. Single nucleotides array was performed. UCSC genome browser was used and gene search was done to find the relevant gene. SPG 4 was identified. Primer was design for the exons of this gene. Primers were ordered and using this forward and reverse primer, Polymerase chain reaction was performed and gel electrophoresis was done for amplification. Amplified product was send to Europhins Bangalore for sequencing. Than sequence were subjected for analysis. But no polymorphism was found during the analysis.

**Results:** In the single nucleotide array we found SPG4 gene which was associated with hereditary spastic paraplegia. We were unable to detect any polymorphism in patient affected with hereditary spastic paraplegia.

**Conclusion:** This study provides evidence that a heritable factor can causes paraplegia in some families and reiterates the importance of recessive genes as a cause of hereditary spastic paraplegia in this part of India.

**Key words:** Hereditary Spastic Paraplegia, Polymorphism, DNA, Exons, Pedigree, Gene.

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### I. Introduction

Hereditary spastic paraplegias (HSPs) are a group of neurodegenerative disorders that share the common feature of lower limb spasticity and weakness. The pathophysiologic trademark of the disease is a “dying-back” axonal neuropathy that mainly affects the upper motor neurons of the corticospinal tracts<sup>1</sup>. As a group, HSPs are rare with predominance estimates extending between two to ten per 100,000 people<sup>2,3</sup>. Though HSPs are clinically greatly varied, most if not all of the many genetic subtypes share some clinical features. Spastic paraparesis is an early and severe feature of the phenotype. If spasticity of the upper limbs is present, lower limb spasticity commonly leads upper limb association by some years. Symptoms progress constantly over years and decades without considerably shortening life expectancy, true at least for pure forms of the disease. In pure HSP, symptoms are mainly limited to the consequences of corticospinal tract dysfunction. Spastic paraparesis may be associated with mild dorsal column sensory discrepancies and bladder disorders. In complex HSP, other neurologic and non-neurologic signs occur, representing a more extensive degenerative mechanism. Common so called complicating indications consist of seizures, amyotrophy, cerebellar atrophy, optic atrophy, peripheral nerve involvement and cognitive deficits<sup>5,13</sup>. Since the first HSP locus was described in 1986.<sup>6</sup> More than 70 HSP loci have been mapped. They are termed SPG1–SPG70 (spastic paraplegia gene 1–70). HSPs can be inherited in an autosomal dominant, autosomal recessive or X-linked pattern. Almost 55 HSP genes have been identified; the associated proteins play important roles in intracellular trafficking, mitochondrial metabolism, and myelination etc.<sup>7</sup>

Phenotypes tend to be rather variable in most HSP subtypes. Age of onset, for example, may vary by several decades even within families. The presence of complicating symptoms or certain MRI abnormalities is a variable feature in many subtypes. This limits the predictability of a certain genotype based on the phenotype. Due to the variability of age of onset, frequent occurrence of de novo mutations and reduced penetrance in some cases, even determining the inheritance pattern may be challenging in HSP.<sup>4</sup> Due to variability in phenotype, mode of inheritance and frequency are often the best guides in choosing the genetic testing strategy for HSPs. The most common form of autosomal dominant HSP is SPG4, accounting for ~50% of cases, followed by SPG3 and SPG31.<sup>12</sup> SPG3 is especially common in early onset cases. Silver syndrome typically occurs in SPG17; however, this has been found in SPG4, SPG10, and SPG31 in rare cases as well. If no male-to-male transmission is observed when taking the family history, X-linked HSPs are also a possibility.<sup>11</sup> In previously reported outpatient clinic, ~40% of cases fall into this category. Careful education of patients about the likely hereditary origin of their disorder and potential risk of transmission to the next generation is of particular importance in these cases. The most common cause of apparently sporadic pure HSP is *SPG4* mutations, occurring in ~10 to 15% of cases. *SPG7* mutations are observed in up to 7%, *SPG5* mutations are probably less common (<5%). This group is rather heterogeneous and difficult to grasp. If cognitive impairment and thin corpus callosum are present, the chance of finding mutations in the *SPG11* gene is almost 60%; if SPG11 is negative, consider testing of the *SPG15* gene (almost 30% in SPG11 negative cases).<sup>11</sup>

The aim of this study is to determine genetic polymorphisms of SPG4 gene in Hereditary Spastic Paraplegia patients. These relations have been proven in western countries but not in the Indian Subcontinent. The purpose of this study is to find new or existing polymorphisms in SPG4 gene which can lead to opening of a new horizon in management of hereditary spastic paraplegia. This study may provide basis for genetic counseling of Hereditary Spastic Paraplegia patients.

## II. Methodology

The present study was carried in the Department of Anatomy, Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, over a period of 2 years starting from March 2014 to June 2016. It was a cross sectional hospital based study. Ethical approval was obtained for the study. A written informed consent was taken by the patient mentioning the pros and cons of the study. The patient and their guardians were given full opportunity to discuss about the study and had full right to quit anytime from the study when desired.

### Inclusion Criteria:

1. Patients with neurological conditions of known molecular aetiology will be genotyped in order to diagnose them molecularly and investigate the phenotype-genotype correlation.
2. Patients with a disease of unknown or incomplete genetic characterisation will be studied with hopes of contributing to the identification of specific genes responsible for that disease.
3. The general health and well-being of each potential participant must be sufficient to allow attending the hospital and allowing blood drawing as indicated.
4. In particular, families with more than one affected individual or families with known consanguinity will be sought, as the incidence of a recessive disorder is increased in this setting.
5. For positional cloning and the homozygosity study, samples from healthy controls from the family will be co-opted.

### Exclusion Criteria:

1. Patients with history of trauma, use of steroids, radiation therapy, laser therapy, posterior segment pathology were excluded from the study.
2. Patient receiving treatment for some disease.
3. Patients with coexisting morbid illness or too ill to participate.
4. Patients with mental incapacity.
5. Patients without suspected inherited neurological disorder.

All patients underwent thorough clinical neurological examination based on pre-determined Performa. Data collected from the patients' records included patients' age, gender, duration of condition, age at onset of condition, presence or absence of other associated complaints, use of any medication, antenatal, natal and perinatal history, developmental history and immunization history. All patients undergone biological workup including complete blood count, renal function test, serum electrolytes, liver function test, blood sugar (both fasting and post prandial). Sampling was done after taking history of condition and detail clinical neurological examination. Genetic study was done with the sample collected from these patients. The pedigree chart was drawn to know about the phenotype present in the family. The pedigree chart must comprise of at least three generations. 4-5ml blood sample will be taken for genetic study by vein puncture from the participants (trio/duo approach). Blood sample of patient and their parents were collected in EDTA/heparin coated viols and if

possible from ancestors of one generation. Unaffected family members who volunteer will be accepted as controls clinical phenotype will be reported to aid in disease classification and identification. After collection of blood DNA was isolated using salting out method. Illumina Single nucleotide polymorphism (SNP) array was done. It is a type of DNA microarray which is used to detect polymorphism within a population. A Single nucleotide polymorphism (SNP), a variation at a single site in DNA, is the most frequent type of variation in the genome. Two restriction sites were identified from SNP array and were paste on UCSC genome Browser available online. Then Gene on Sequence was found. We identified three genes which may be responsible for the phenotype of hereditary spastic paraplegia. SPG 4 is located on Chromosome 2p21-2p22, it functions as a microtubule severing protein & regulates ER morphology, it encodes spastin protein, it is a member of adenosine triphosphatase.

**Procedure for Primer Designing:** The gene sequences were obtained from the Ensemble Genome Browser (GRCh38 assembly, Feb 2009) ([http://www.ensembl.org/Homo\\_sapiens/Info/Index?db=core](http://www.ensembl.org/Homo_sapiens/Info/Index?db=core)). Primers used for PCR amplification were designed using Primer3 software version 0.4.0 (<http://frodo.wi.mit.edu/primer3/>). Primer sizes were 20-24 bases in length (the specificity and annealing temperature are dependent on the length of the primer). Melting temperatures were kept as similar as possible for both the left and right primers to maintain PCR efficiency (ideally between 59-62°C). Where possible the guanine – cytosine (GC) base content was kept between 40-60%. Primers with inter or intra-primer efficiency extending for more than 3 bases were avoided, to prevent the formation of secondary structures and primer dimmers. The primer sequences selected were specific and a 100% match to the region to be amplified. In silico PCR analysis and Blast searches were performed using the UCSC Genome Bioinformatics website (website <http://genome.ucsc.edu/>) to check for the design. The primers were ordered for those exons which were found to be affected most commonly according to review of the literature. Primer was design for the exons 7,8,9,10,11,12,13,15,16 and 17 of spg4 Then primers were ordered all these exons. Primers were diluted according to their yield mentioned on the tubes and was kept for 24 hours to get diluted properly. Then annealing temperature was decided according to polymerase chain reaction product and gel electrophoresis results .Temperature showing best band with maximum brightness and thickness was taken as annealing temperature. Polymerase chain reaction was done for all the premiers and gel electrophoresis was carried.

**Table 1.List of primers**

EXONS OF GENE SPG4	FORWARD PRIMER & TEMP	REVERSE PRIMER & TEMP	PRODUCT SIZE
exon 7	GCCAGGCTGGTCTTTCATAC 59.52	CCTCCTGGTTC AAGCAATTC 59.70	203
exon 8	TTTTACCACCGAGGAGTTGG 59.96	TCACCTGAGCTCCAGCTAT 59.80	151
exon 9	CGTGAGTGCTCTGTTTCCAA 60.02	TTAAGCCAGCCAGTTTACGG 60.26	239
exon 10	CTCTCCCCTTCTCAAACCA 59.92	ACAGCAAAAAGAGCCCTCAC 60.12	239
exon 11	AAAGAAGGGCAAGCTTAAAGAC 58.93	ATCGTGCTCCCCTTCTCTTC 60.73	233
exon 12	GCTTGATGAGGCTGTTCTCA 60.00	ACCAATTTGCCTAAAACCGTAA 59.03	248
exon 13	GCTTTTCTGTCATTTGCTG 58.50	GCTGTACCATGGATTGGAAGA 59.95	209
exon 15	ACTACAGGCATGCACCATCA 59.98	TTGCCTAGAACAGGGGTAC 60.23	173
exon 16	GTACCACCAAGCCAGCTAA 60.34	AGTCTGGGCGACAGAGTGAG 59.68	250
exon 17	GAGACCCTGTTTCTGCCAAA 60.23	TGATCTTGGCTCACTGCAAC 59.99	157

**DNA quantification :** It was done for the DNAs ,isolated from the blood samples collected for genetic study to know the concentration of DNA in the sample, by the help of nano drop so that we can get the proper bands in gel electrophoresis. And those who were having concentration of 1 nano drop or more than one nano drop were included for PCR. The PCR was carried out on a Applied Biosystem, 96 well Veriti Thermal Cycler (Applied Biosystem, USA).

**Table 4: Procedure for PCR**

Step	Segment	Temperature(°C)	No. of cycles	Time for each segment (in sec)
1	Denaturation	95 °C	35	45 sec
2	Annealing	Temperature Dependent	35	45 sec
3	Extension	72 °C	35	45 sec

**Table 3- Reaction Mixture**

Sl. No.	Reagents	Amount (Per reaction)
1.	Taq buffer	2µl
2.	Forward primer	(5 pmol/µl) 1.5 µl
3.	Reverse primer	(5 pmol/µl) 1.5 µl
4.	Mili Q water	13.3 µl
5.	Dntp	0.5µl
6.	Taq polymerase	0.2 µl
7.	DNA µl)1µl	(100ng/
Total Volume		20.0 µl

The product of PCR were subjected to agarose gel electrophoresis for amplification. 5µl PCR amplified products were loaded with DNA loading dye (Bromophenol blue) to check the amplification. Following procedure was followed for gel electrophoresis. Edges of the plastic tray were sealed with tape to form a mould. The tank was filled with sufficient amount of TAE buffer (50X). 2 gram of agarose was dissolved in 100ml of buffer and the solution was heated. The molten gel was allowed to cool and Etbr (Ethidium bromide) was added to a final concentration of 0.5µg/ml. The gel solution was mixed thoroughly by gentle swirling. Combs were placed over the plate so that a complete well was formed when the agarose was added to the mould. The gel was allowed to solidify and the samples were then loaded at 100 Voltage till the bands were completely separated. The gel was then placed in UV trans-illuminator to observe the DNA bands. The amplified product which showed proper bands on electrophoresis were sent for DNA sequencing using the chain termination method developed by Frederick Sanger. For sequencing, the PCR products were outsourced to Eurofins at Bangalore. DNA sequencing was performed in both directions, initiated from the forward and reverse PCR primers. After that, analysis was done in dry lab using Finch TV (<http://www.geospiza.com/ftvdlinfo.html>), ClustalX2<sup>7</sup> and MEGA6.

**OBSERVATION AND RESULTS**

These are the peaks showing results of sequencing of one family included in this study.

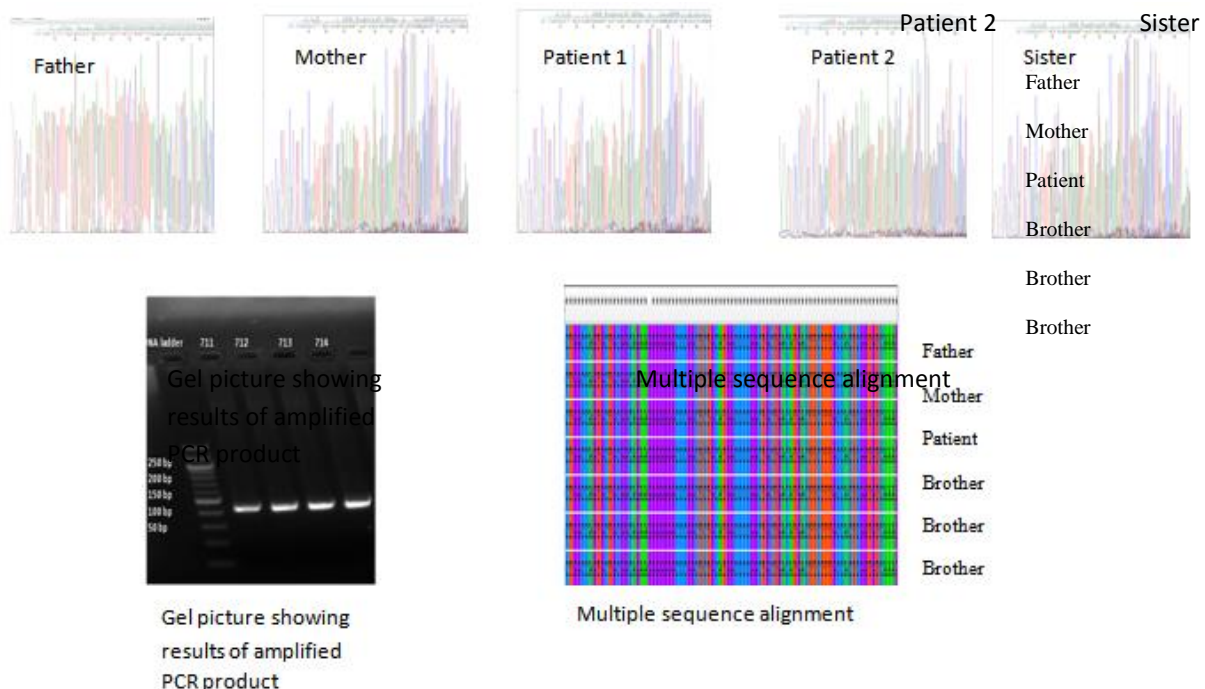
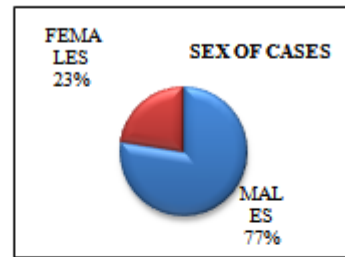


Table 1 showing Mean ± STDEV of male & female with hereditary spastic paraplegia collected from the hospital database. Hereditary Spastic Paraplegia 11males (77%) & 6 (23%) females, sex ratio of 1:1.5 .Pie chart 1 showing percentage of male and female

Table 1.Number and Mean ± STDEV of male & female

Sex	Number	Mean ± STDEV
Male	11	4.3±0.888194
Female	6	4.75±1.258306

Pie chart 1.Showing percentage wise distribution



Bar diagram 1.Showing religion wise distribution among the HSP cases

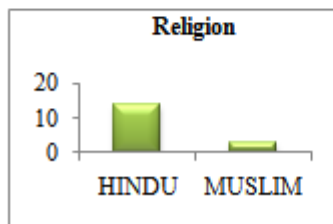
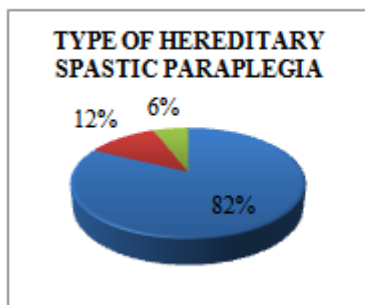


Table 2.Showing number of cases according to the type of HSP

Types Of HSP	No.
pure	14
complex	2
Associated with others	1

During the study according to religion patients were categorized into two categories Hindu (14) and Muslims (3) as shown in bar diagram 1. We observed that maximum patients suffering from Hereditary Spastic Paraplegia were Hindu in this part of country and also were of upper caste and Brahmins. The study also includes the type of hereditary spastic paraplegia during two years period as shown in table 2. The type of hereditary spastic paraplegia included pure, complex and associated with other condition shown in pie chart 2. During the period pure (14 or 82.3%), complex (2 or 11.7%) and associated with other condition (1 or 5.8%) were major type of hereditary spastic paraplegia encountered in our study.

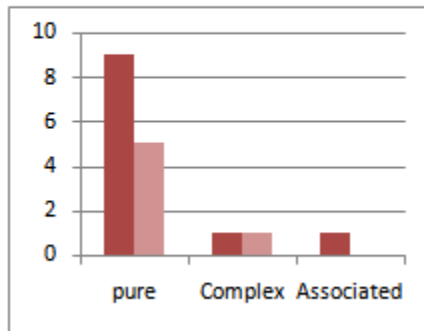
Pie chart 2.Showing types of HSP



Type of HSP	Male	Female
pure	9	5
Complex	1	1
Associated	1	

Pie chart 2 showing type of hereditary spastic paraplegia in the number of cases collected from Sir Sunderlal Hospital.As the percentage of pure Hereditary Spastic Paraplegia was maximum in our study. We checked distribution pattern of Hereditary Spastic Paraplegia according to sex as in table 3. Percentage of male candidate was maximum in Hereditary Spastic Paraplegia as compared to females in our study shown in bar diagram 2. Study demonstrates age of presentation of cases in which majority were between 12 – 18 years (10, 58.8%). 2 out of 17 cases were of below 15 yrs of age & 15 were greater than 15 years of age shown in bar diagram 3.

3. Bar diagram 2. Showing gender distribution among the types of HSP



Bar diagram 3 .Showing age distribution

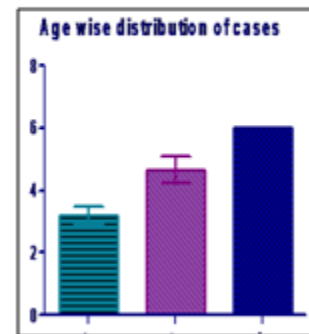


Table Demonstrating distribution of type of Hereditary Spastic Paraplegia according to the gender of the cases.

### III. Discussion

Seven families with Hereditary Spastic Paraplegia from a Sir Sunderlal Hospital were included in the present study. All patients were neurologically and genetically evaluated after giving informed consent. Genomic DNA was extracted from peripheral blood leukocytes. All coding exons of spg4, spg10 and spg11 were amplified by polymerase chain reaction using primers designed Sequencing was performed by Eurofins at Bangalore .Mutations detected were analyzed with reference to the Human Gene Mutation Database and any novel mutations not reported in the database were confirmed by sequencing of 100 control Among the 18 probands, the disease was inherited as AD in 11 and was sporadic in 7. The first presenting symptoms in the patients were weakness and/or spasticity; 12 of 18 patients had weakness and spasticity, 1 patient had only weakness, and 5 patients had only spasticity. The age at onset of symptoms showed a wide range, from 1 to 59 years (mean  $\pm$  SD,  $34.6 \pm 15.8$  years). All patients were diagnosed as having uncomplicated HSP characterized by spasticity and hyperreflexia of the lower limbs. Nine patients had accompanying bladder function disturbances, and 4 patients showed decreased vibratory sense in the lower extremities without any other sensory impairment. Most patients, except for 2 who displayed hyperreflexia of the arm, exhibited no upper extremity symptoms. The disability status of the patients also showed a considerable variability. The disability staging could not be assessed in 2 children who had not started walking (patients 3 and 5). It was a Cross sectional hospital based study which was done involving the patient from different parts of the eastern Uttar Pradesh that were referred for treatment in Sir Sunderlal Hospital, Banaras Hindu University . In our study, it was observed that during the last 2 year male to female ratio was 2:1. However, the ratio is very high this may be due to an underestimation of the true situation since; in India many people do not come to hospital if a girl child has this type of disease. This needs a door to door study of a location. In our study we did observe that the occurrence of uncomplicated hereditary spastic paraplegia was most prevalent as compared to complicated hereditary spastic paraplegia & that of the familiar history with complicated hereditary spastic paraplegia were least. We conclude that uncomplicated hereditary spastic paraplegia with family history was the most common type of hereditary spastic paraplegia as also demonstrated in other studies.<sup>14</sup> Distribution according to religion showed the maximum number of affected patients belong to Hindu religion as in the Indian scenario majority belongs to Hindu community as compared to Muslim community. Study involving more number of cases from the general population may confirm this finding. Majority of our cases had normal motor developmental milestones. This is due to fact that hereditary spastic paraplegia mostly demonstrates in the age of puberty and the different types of distribution of spasticity & weakness with the variable involvement of different parts of the body but in most of the cases only involvement of lower limbs is apparent. The Upper limb seems to be normal. One of them who had involvement of upper limbs, bowel and bladder and also cognitive impairment had complicated hereditary spastic paraplegia which was less common among the population of eastern Uttar Pradesh. Age of onset can be from infancy to eighth decade marked interfamilial variations in the age of onset was one of the early pointers to genetic heterogeneity in this condition.<sup>15</sup> This variation may also be partly due to difficulty in ascertaining an exact date of onset, particularly in the older patients who have had disease for decades, or may reflect other as yet unknown genetic factors. Very little work has been performed to correlate an association between SPG4 gene and hereditary spastic paraplegia. SPG 4 plays an important role in function of microtubule severing protein and regulate ER morphology .It encodes spastin protein, a member of adenosine triphosphatase. It is emphasized that it may play an important role in development of muscles too. We could not

demonstrate any polymorphisms of SPG4 gene in our subjects possibly due to smaller sample size and less familial cases. But further work in this study is required and may possibly let us a peep into the role of SPG 4 in the development of muscle its structure and tone and its association with the hereditary spastic paraplegia in this part of the country. No mutations of the SPG4 gene were detected.<sup>16, 17, 18, 19</sup> None of the novel variants were observed in 100 control chromosomes by direct sequencing of the corresponding exons. Seven patients with no mutations had 1 or more affected members in the family, suggesting an AD inheritance pattern, and 1 patient had no family history of HSP. No mutations were detected in encoding region of the SPG4 gene. Molecular genetic analysis of the SPG4 genes in 7 unrelated eastern Uttar Pradesh families and adjoining areas of Bihar patients with uncomplicated HSP revealed no mutations in the SPG4 gene.<sup>20</sup> Of the 11 patients with a familial background of AD inheritance, 7 had no SPG4 mutations. Possible explanations no SPG4 mutation detection in our series include (1) the small number of cases in the present study; (2) different methods of mutation detection: direct sequencing analysis in the present study vs. mutation screening in the previous reports; and (3) an ethnic difference in the genetic background of AD-HSP. Among 7 patients without any family history (sporadic cases), no mutation detection was noted, in line with previous studies.<sup>21</sup> Nevertheless, mutation analysis of the SPG4 gene in patients with sporadic spastic paraplegia is of significance in the diagnosis and genetic counseling of patients and family members because identification of the mutation can confirm the diagnosis of spastic paraplegia, and patients with sporadic spastic paraplegia with an SPG4 mutation have the same chance of passing the mutation to their offspring as patients with AD-HSP. Although approximately 10% of uncomplicated AD-HSP is known to be caused by mutations in the SPG4 gene, none of our patients had SPG4 gene mutations. This might be attributable to a small number of patients with AD-HSP who did not have the SPG4 mutation in the present study or to an ethnic difference. It is necessary to recruit more patients with AD-HSP to investigate the contribution of SPG4 mutations in AD-HSP in eastern Uttar Pradesh and adjoining areas of Bihar. Although most reports of SPG4 mutations show a significant proportion of splice-site mutations (30%-50%), our study revealed no mutation (12.5%), since we used primers that covered approximately 60 base pairs of the exonic regions, mutations in these sites could not be missed.

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