SCREENING OF SPASTIN, ATLASTIN, NIPA1 AND REEP1 GENES IN HEREDITARY SPASTIC PARAPLEGIA PATIENTS

by

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B.S., Medical Biology, Istanbul University, 2006

Submitted to the Institute for Graduate Studies in
Science and Engineering in partial fulfillment of
the requirements for the degree of
Master of Science

Graduate Program in Molecular Biology and Genetics

Boğaziçi University

2008

ACKNOWLEDGEMENTS

First of all, I would like to express my special thanks to my supervisor Assoc. Prof. Esra Battaloğlu for her unlimited support, guidance and encouragement throughout the study.

I also would like to thank other members of thesis committee, Prof. Hande Çağlayan and Prof. Yeşim Parman, for giving their valuable time in order to evaluate my thesis.

I would like to appreciate to Prof. Yeşim Parman and Dr. Nihan Hande Akçakaya for providing blood samples and clinical data of patients used in this study.

I would like to express my sincere gratitude to my friend Duygu Dağlıkoca for helping and advising me whenever I needed. I am also grateful to Seda Salar and İrem Akat for their friendship and support. I would like to extend my thanks to all my friends in RECEP laboratory; Birdal Bilir, İbrahim Barış, Başak Şenergin, Gamze Küser, Cihan Erkut, Demet Candaş, Neslihan Zohrap and Mahmutcan Hız, and all my friends in the MBG Department.

I specially thank İlyas Okur for his continuous moral support and invaluable friendship.

Last but not least, I would like to express my biggest thank to my family for understanding me all the time and encouraging me to pursue my interests.

Finally, I am grateful to TUBITAK-BIDEP 2228 for awarding and supporting me during my master thesis.

ABSTRACT

SCREENING OF SPASTIN, ATLASTIN, NIPA1 AND REEP1 GENES IN HEREDITARY SPASTIC PARAPLEGIA (HSP) PATIENTS

Hereditary Spastic Paraplegia (HSP), also known as Strümpell-Lorrain Syndrome or Familial Spastic Paraplegia (FSP), is a group of inherited neurodegenerative disorders that is characterized by progressive spasticity and weakness of the lower extremities. The prevalance of the disease is three people per 100,000. HSP is a genetically heterogeneous disease and up to date, 39 loci for autosomal dominant, recessive, and X-linked forms of HSP have been identified.

In this study, the genetic background of HSP disease was investigated in a total of 42 HSP and CMT patients. Since *Spastin*, *Atlastin*, *REEP1* and *NIPA1* mutations are responsible for more than 50 per cent of AD-HSP cases, these genes were screened for probable sequence alterations by SSCP analysis. Although Spastin gene mutation is known to be the most common (about 40 per cent) genetic defect leading to SPG4 phenotype in other populations, only two Spastin mutations were found among 13 AD pure HSP patients. Consistently, Atlastin mutation, the second most common (about 10 per cent) genetic defect resulting in SPG3A phenotype, was found only in one patient. Besides, no mutations on NIPA1 and REEP1 genes could be identified suggesting that genetic contribution of these genes in our population is not very high.

The missense Atlastin mutation, c.941A>G, identified in one HSP patient may lead to the disease by impairing the GTPase activity of the protein via introducing an abnormal secondary structure, or association of atlastin with other proteins such as spastin. Besides, an insertion (c.310_311insA) and a nonsense (c.1741C>T) mutation were identified in the Spastin gene of two of our patients, resulting in formation of truncated proteins. These alterations may directly cause loss of protein activity by inactivating functional AAA domain which in turn leads to decrease the amount of

functional spastin. These findings will help to unravel the possible functions of Atlastin and Spastin genes in the pathogenesis of AD pure HSP.

The patients should be further screened for other AD-HSP genes. Furthermore, according to the high number of consanguineous marriages in Turkey, these results may be the indicator of high incidence of autosomal recessive types of HSP in Turkish population.

ÖZET

HEREDİTER SPASTİK PARAPLEJİ (HSP) HASTALARINDA SPASTİN, ATLASTİN, NIPA1 AND REEP1 GENLERİNİN TARANMASI

Strümpell-Lorrain Sendromu veya Ailesel Spastik Parapleji olarak da bilinen Herediter Spastik Parapleji (HSP), alt ekstremitelerde görülen ilerleyici kasılma ve zayıflık ile karakterize edilen kalıtsal nörodejeneratif bir hastalık grubudur. Hastalığın görülme sıklığı 100.000 kişide üçtür. Genetik çeşitlilik gösteren bir hastalıktır ve şimdiye kadar otozomal baskın, otozomal resesif ve X'e bağlı kalıtılan HSP tiplerinden sorumlu 39 kromozomal bölge tanımlanmıştır.

Bu çalışmada, 42 HSP ve CMT hastasında HSP'nin genetik altyapısı araştırıldı. *Spastin*, *Atlastin*, *REEP1* and *NIPA1* mutasyonları otozomal baskın HSP vakalarının yaklaşık yüzde 50'sinden sorumludurlar ve bu sebeple, hastalarımızın taşıdıkları olası dizi değişimlerinin saptanabilmesi için SSCP analizi kullanılarak incelendiler. SPG4 fenotipine yol açan Spastin gen mutasyonları diğer toplumlarda en yaygın genetik bozukluk olarak bilinse de (yaklaşık yüzde 40), bizim hasta grubumuzda sadece iki hastada belirlendi. Aynı şekilde, ikinci en yaygın genetik hasar belirlenen (yaklaşık yüzde 10) ve SPG3A fenotipiyle sonuçlanan Atlastin mutasyonuna hasta grubumuzda sadece bir hastada rastlandı. NIPA1 ve REEP1 genlerinde mutasyon belirlenmemesi bu genlerin ülkemizdeki HSP vakalarına genetik katkılarının düşük seviyede olabileceğini göstermektedir.

Hastalarımızdan birinde Atlastin geninde tanımlanan c.941A>G yanlış anlam mutasyonunun anormal sekonder yapıya neden olarak proteinin GTPaz aktivitesini bozabileceği veya atlastin proteininin diğer proteinlerle bağlantısını engelleyebileceği savlandı. Bunun dışında, iki hastamızda Spastin geninde kısa protein oluşumuna yol açan

bir insersiyon (c.310_311insA) ve bir nonsense (c.1741C>T) mutasyonu bulunmuştur. Bu değişimler fonksiyonel AAA bölgesini bozarak fonksiyonel spastin miktarının azalmasına ve doğrudan protein aktivitesinin düşmesine neden olabilirler. Elde ettiğimiz bu sonuçlar, Atlastin ve Spastin genlerinin otozomal baskın saf tip HSP patogenezindeki olası rollerinin anlaşılmasına yardımcı olacaktır.

Mutasyon saptanmayan hastalarda otozomal baskın HSP'den sorumlu diğer genlerin taranması gerekmektedir. Bunun dışında, ülkemizdeki akraba evliliklerinin sayısının fazlalığı da göz önüne alındığında, hasta grubumuzda otozomal resesif HSP tiplerinin diğer toplumlara oranla daha yaygın olabileceği sonucu doğmaktadır.

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A	Adenine
C	Cytosine
E	Glutamic acid
G	Guanine
H	Histidine
N	Asparagine
P	Proline
R	Arginine
T	Thymine
X	Stop codon
Y	Tyrosine
aa	Amino acid
AAA	ATPases associated with various cellular activities
AD	Autosomal dominant
AFG3L2	AFG3 ATPase family gene 3-like 2
Ala	Alanine
APS	Ammonium persulfate
AR	Autosomal recessive
ARL2	ADP-ribosylation factor-like 6 interacting protein 2
ARSACS	Autosomal Recessive Spastic Ataxia of Charlevoix-Saguenay
ARSAL	Autosomal Recessive Spastic Ataxia with Leukoencephalopathy
AS	Angelman syndrome
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BSCL2	Berardinelli-Seip congenital lipodystrophy type 2
bp	Base pair
BPB	Bromophenol Blue
CAAX	C is Cysteine, A is aliphatic, and X is a variety of amino acids
CAM	Cell adhesion molecule

CGL	Congenital generalized lipodystrophy
cM	Centimorgan
cm	Centimeter
CMT	Charcot-Marie-Tooth disease
CNS	Central nervous system
COS-7	African green monkey SV40-transformed kidney fibroblast cell line
Cpn10	10 kDa chaperonin
Cpn60	60 kDa chaperonin
CYFIP1	Cytoplasmic FMR1 interacting protein 1
del	Deletion
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
E. Coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
EtBr	Ethidium bromide
EtOH	Ethanol
FSP	Familial spastic paraplegia
GBP	Guanylate binding protein
GCP5	Gamma complex associated protein 5
Glu	Glutamic acid
Gly	Glycine
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
HSP	Hereditary spastic paraplegia
Hsp10	Heat shock protein 10
Hsp60	Heat shock protein 60
Ig	Immunoglobulin
ins	Insertion
KCl	Potassium chloride
kDa	Kilodalton
KIF5A	Kinesin family member 5A

LICAM	L1 cell adhesion molecule
m-AAA prot.	Membrane bound ATP-dependent protease
Mb	Megabase
$MgCl_2$	Magnesium chloride
MIT	Microtubule interacting and endosomal trafficking
miRNA	Micro RNA
MLPA	Multiplex ligation-dependent probe amplification assay
MN-1	Meningioma-1
mRNA	Messenger RNA
MT	Microtubule
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NES	Nuclear export signal
ng	Nanogram
NIPA1	Non imprinted in Prader-Willi/Angelman syndrome 1
NIPA2	Non imprinted in Prader-Willi/Angelman syndrome 2
NLS	Nuclear localization signal
nm	Nanometer
PCR	Polymerase chain reaction
PLP1	Proteolipid protein 1
PMD	Pelizaeus–Merzbacher disease
PNP	Peripheral polyneuropathy
PNS	Peripheral nervous system
PWS	Prader Willi syndrome
REEP1	Receptor enhancing-expression protein 1
rpm	Revolution per minute
SAX2	Spastic ataxia 2
SCA	Spinocerebellar ataxia
sec	Second
SDS	Sodium dodecyl sulfate
SNX15	Sorting nexin 15
SPAST	Spastin gene
SPG	Spastic paraplegia

SPOAN	Spastic paraplegia, optic atrophy, and neuropathy
SSCP	Single Strand Conformation Polymorphism
TE	Tris-EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
TBE	Tris-Boric acid-EDTA
TMD	Transmembrane domain
UTR	Untranslated region

1. INTRODUCTION

Hereditary Spastic Paraplegia (HSP), also known as Strümpell-Lorrain Syndrome or Familial Spastic Paraplegia (FSP), is a clinically and genetically heterogeneous group of inherited neurodegenerative disorders (Tallaksen *et al*., 2001). The disease was first described by a German neurologist, Adolph Strümpell in 1883 and defined more comprehensively by a French physician, Maurice Lorrain in 1888. The prevalance was accepted as about three people per 100,000 (Filla *et al*., 1992; Leone *et al.*, 1995; Silva *et al.*, 1997; McDermott *et al*., 2000).

The essential clinical features of HSPs are spasticity and weakness of the lower extremities, bladder disturbances, and mild vibratory sensory impairment. Although progressive spastic weakness is commonly seen in several inherited disordes such as multiple sclerosis, Alzheimer's disease, Friedreich's ataxia and Spinocerebellar ataxia (SCA), HSPs can be discriminated from these diseases by the occurrence of other related neurological manifestations (Fink, 2003a; Fink, 2003b). Neuropathological examinations of pure HSP cases showed the presence of length-dependent, distal axonal degeneration that primarily affects the upper motor neurons (Behan and Maia, 1974; Harding, 1983; Deluca *et al*., 2004; Evans *et al*., 2006). The upper motor neurons control lower extremity motor function and are one of the longest neurons in the central nervous system. Their axons' length can be larger than one meter and constitute 99 per cent of the total cell volume. Conversely, lower motor neurons with long axons, such as the anterior horn cells, are generally protected in the pure types of HSPs (Reid, 2003a; Reid, 2003b; Holzbaur, 2004; Soderblom and Blackstone, 2006). In some patients with early-onset HSP, abnormally thin corticospinal tracts were defined suggesting that abnormal development of these tracts may also cause various forms of HSP (Casari and Rugarli, 2001).

The upper motor neurons exhibit a dying back neuropathy in HSP and the damage occurs primarily at the terminal and preterminal regions of the neurons, instead of cell bodies (Figure 1.1) (Gould and Brady, 2004). Several localized neuronal insults influence

both anterograde and retrograde intracellular signals through the path they use. Thus, neurons are specifically affected at the damages of cellular functions including energy usage, synaptic transmission, signaling, cytoskeleton formation, vesicle trafficking, and organelle transport (Crosby and Proukakis, 2002; Soderblom and Blackstone, 2006).

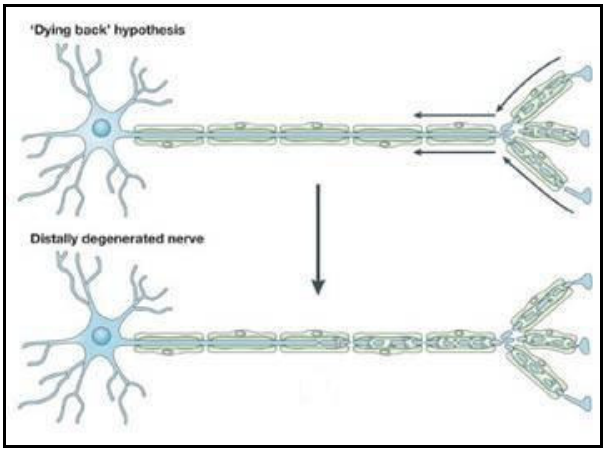


Figure 1.1. The 'dying back' model of axonal degeneration (modified from Coleman, 2005).

Age of symptom onset can vary among HSP patients. Symptoms may appear at infancy or late in life. However, in most of the patients, symptoms start to develop just after adolescence or at the middle age (Soderblom and Blackstone, 2006). Not only symptom onset but also severity and prognosis of HSP can differ between and within families. Approximately 30 per cent of HSP patients are asymptomatic, suggesting that the disease may also have a common benign nature. For this reason, clinical evaluation is very important and genetic studies should be a part of clinical analysis of families (Fortini *et al.*, 2003).

HSPs have been classified as 'pure' or 'complicated'. As implied before, pure forms are characterized by spasticity and weakness of the lower extremities. However, sphincter disturbances, minor vibration sense loss, and minimal cerebellar ataxia of the upper limbs can also be observed as the disease progress (Durr *et al.*, 2004). Complicated types of HSP involve one or more neurological or non-neurological symptoms along with

progressive spastic weakness; such as thin corpus callosum, deafness, epilepsy, amyotrophy, ataxia, mental retardation, and optic retinopathy (Harding, 1981).

Pure type of HSP can be divided into two subgroups based on the age of symptom onset. If the patient's age of onset is below 35 years, the disease is accepted as type I, while type II is characterized by onset over 35 years. In type I cases, delayed walking is common and spasticity of the lower limbs is more apparent than weakness. On the other hand, type II pure HSP progress more rapidly and muscle weakness, bladder disturbances, and sensory loss are more obvious (Fortini *et al*., 2003).

HSPs are further classified according to the mode of inheritance, which can be autosomal dominant, recessive, or X-linked. To date, 39 loci for autosomal dominant, recessive, and X-linked forms of HSP have been mapped, and 17 proteins have been identified (Meijer *et al*., 2007).

1.1. Autosomal Dominant HSP

1.1.1. SPG3A

SPG3A is a pure form of HSP with early onset and slow progression. Even in some patients, a clear progression can not be detected. Because of these features, abnormalities in the central nervous system development have been proposed as the cause of SPG3A HSP (Hazan *et al*., 1999; Zhao *et al*., 2001; Fink, 2002).

SPG3A locus was identified in 1993 and nearly 15 per cent of autosomal dominant pure HSP cases were found to be associated with this locus on chromosome 14q11-21 (Hazan *et al*., 1993; Fink *et al*., 1996). Subsequent studies reduced this locus to a 2.7 cM interval (Rainier *et al.*, 2001) and candidate gene approach identified the causative gene as *SPG3A* (Zhao *et al.,* 2001). A disease specific missense mutation was detected in affected individuals from five SPG3A-linked kindreds (Zhao *et al.,* 2001). Further studies showed that *SPG3A* is the second most commonly mutated HSP gene and approximately 10 per cent of dominant families carry *SPG3A* mutations (Rainier *et al.,* 2006).

SPG3A is encoding for atlastin protein that is expressed predominantly in the central nervous system. The protein is also detected in other tissues including kidney, testis, smooth muscle, adrenal gland and lung (Zhao *et al.*, 2001; Zhu *et al*., 2003). In SPG3A patients, the protein was particularly found in the lamina V pyramidal neurons in the cerebral cortex. Knock-down studies suggest that atlastin is necessary for axonal growth based on its high levels at growth cones and growth-cone like varicosities throughout the axons, and at branch points in developing rat (Zhu *et al*., 2006). In the cells, atlastin is localized to the Golgi apparatus and ER during neuronal development (Zhu *et al*., 2003; Sanderson *et al.*, 2006).

Atlastin, also called as atlastin-1, is an integral membrane protein with two putative transmembrane domains and its molecular mass is approximately 63.5 kD. It contains three conserved motifs P-loop, DxxG and RD motifs that are the characteristics of guanylate binding/GTPase active sites (Zhao *et al*., 2001). Although it forms homo-tetramers, it is not known whether atlastin-1 makes heteromeric complexes with atlastins (Zhu *et al*., 2003).

Based on nucleotide and protein similarity, atlastin (also known as atlastin-1) was found to be highly related with two other proteins; atlastin-2 and atlastin-3. These proteins also have a GTP binding domain in the N-terminal region that share noticeable homology with atlastin-1 (Zhu *et al*., 2003).

According to phylogenetic analysis, atlastin has an important homology with human guanylate binding protein-1 (GBP1) and mouse guanylate binding proteins (ARL-6 interacting protein-2). Moreover, it also exhibits sequence similarity in its GTPase domain with the human and murine members of the septin family of GTPases; human septin-3 and mouse septin-3 (Zhao *et al*., 2001). Among them, atlastin is particularly homologous to GBP1. It includes an amino-terminal GTPase domain and a following carboxy-terminal helical domain which are analogous to the features of GBP1. Locations and conformations of the P-loop, DxxG and RD motifs of atlastin are similar to the corresponding residues of GBP1 (Zhao *et at*., 2001). In contrast to sharing similar features with GBPs, there are several structural differences. Atlastins do not have an additional C-terminal α-helical domain and a CAAX (A represents an aliphatic amino

acid) isoprenylation motif. Moreover, atlastins are suggested to be integral membrane proteins with two putative transmembrane domains, whereas GBPs are not. In this aspect, Fzo/mitofusins show similarity with atlastins due to being large GTPases. They extend on the outer mitochondrial membrane and their N- and C-terminals protrude into the cytoplasm like atlastin (Zhu *et al*., 2003).

Despite the presence of several predictions for the function of atlastin, its exact role is not entirely known. Due to its similarity with GBP1, atlastin protein is classifed as a member of the dynamin family of large GTPases (Noda *et al*, 1993; Urrutia *et al*., 1997; Schmid *et al*., 1998; McNiven *et al*., 2000). Dynamins are required for proper and efficient vesicle recycling (Jones *et al*., 1998; Nicoziani *et al*., 2000) that is important for the maintenance of synaptic membrane structure and neurotransmission (Vogler *et al*.,1998; Della Rocca *et al*., 1999; Carroll *et al*.,1999; Zhang *et al*., 2000). Moreover, dynamins are essential for the preservation and distribution of mitochondria and are connected with actin and microtubules (Ochoa *et al*., 2000).

Several studies established an association between atlastin and SPG4 causing gene product; spastin. Yeast-two-hybrid and co-immunoprecipitation studies in Hela cells implied that N-terminal domain of spastin bound to C-terminal cytoplasmic domain of atlastin and this interaction was not distrupted by a mutation at the N-terminal GTPase domain of atlastin. This finding suggested that GTPase activity of atlastin does not have a role in spastin-atlastin interaction (Evans *et al.*, 2006; Sanderson *et al*., 2006). These results suggested that absence of proper spastin-atlastin interaction may be responsible for the disease at the molecular level.

To date, more than 20 different mutations have been identified that are distributed throughout the gene. Most of them were missense alterations but also an in frame deletion and a nucleotide insertion in exon 12 (c.1520insA) producing a premature protein was defined (Tessa *et al.,* 2002). These mutations were shown not to affect mRNA expression levels of the SPG3A gene suggesting that protein stability rather than mRNA expression or stability was affected. Furthermore, an in frame deletion (p.del436N) did not impair the spastin-atlastion interaction or GTPase activity of atlastin,

but it reduced atlastin levels. In this regard, this mutation was suggested to create both a loss of function or dominant-negative phenotype (Meijer *et al.*, 2007).

To sum up, while some mutations are suggested to show a pathogenic effect by impairing multimerization, by changing secondary structure of the protein, or damaging interactions of atlastin with other proteins, some are thought to damage its GTPase activity directly (Zhao *et al.,* 2001; *Meijer et al*., 2007).

1.1.2. SPG4

The SPG4 gene is known to be mutated in at least 40 per cent of all autosomal dominant HSP families. Most of these patients show pure HSP phenotype, with remarkable variation in age at onset and severity of the disease. On the other hand, several *SPG4* mutations may result in complicated type of HSP as seen in some families with late-onset dementia. Besides, atypic cortical pathology including tau-immunoreactive neurofibrillary tangles in the hippocampus and tau-immunoreactive balloon cells in the limbic area and neocortex was detected in one affected individual (White *et al*., 2000).

Genetic linkage studies among several families from different ethnic groups has led to map the AD-SPG4 locus to chromosome 2 (Durr *et al*.,1996). A candidate gene, SPG4 (*Spastin)* has been located on chromosome 2p22-p21 in 1999. The gene includes 17 exons extending over 90 kb region and encodes for spastin protein (Hazan *et al*., 1999).

Based on protein sequence analysis, spastin is accepted as a member of the subfamily-7 group of proteins called (AAA) ATPases associated with various cellular activities. This group also includes the p60 subunit of katanin, the mouse SKD1 protein, the yeast Yta6p and Sap1, and three fidgetin proteins. The AAA proteins are important for different cellular processes such as cell cycle regulation, organelle biogenesis, membrane trafficking, microtubule regulation and protein degradation in which they are suppossed to behave as chaperons (Patel *et al*., 1998; Neuwald *et al*., 1999; Salinas *et al*., 2007).

The spastin protein is composed of 616 amino acids and includes two essential domains; a microtubule interacting and endosomal trafficking (MIT) domain at the N-terminus and the AAA domain at the C-terminus (Hazan *et al*., 1999; Salinas *et al*., 2007). The MIT domain is highly conserved through diverse proteins such as Vsp4, spartin, sorting nexin 15, and katanin and responsible for the spastin-microtubule connection (Takasu *et al*., 2005). The AAA domain is also represented in another AAA family member; paraplegin that is known to be responsible for SPG7. However, spastin does not include the paraplegin domains that serve as metalloprotease and mitochondrial sorting peptide, suggesting that they belong to different subgroups of AAA proteins (Casari and Rugarli, 2001).

The AAA cassette is localized between amino acids 342 and 599 and is responsible for the ATPase activity of protein (Evans *et al*., 2005). The three conserved ATPase domains, Walker motifs A and B, and the AAA minimal consensus were located in this region. Walker motif A responds to the ATP-binding domain and together with Walker motif B, they are highly conserved between spastin and all AAA family members (Hazan *et al*., 1999). Additionally, spastin has two functional nuclear localization signals (NLS), two nuclear export signals (NES) and a putative transmembrane domain (Beetz *et al*., 2004; Claudiani *et al.,* 2005).

Spastin is commonly expressed in neuronal and non-neuronal tissues. Alternative splicing mechanism and use of diverse translation codons cause generation of several spastin isoforms in the cells. The highly conserved 5' untranslated region (5'- UTR) was shown to control the preferential use of different ATGs and two isoforms translated from the SPG4 mRNA were localized to different intracellular compartments confirming the data given by previous immunolocalization studies (Charvin *et al.,* 2003; Wharton *et al*., 2003; Beetz *et al.*, 2004; Errico *et al*., 2004). The longer isoform contains an N-terminal NES motif and is exported from the nucleus, whereas the shorter isoform has both nuclear and cytoplasmic localization. In some cell lines and rat brain, short isoform is partially translated. According to another study, absence of the regulatory 5' UTR in spastin-encoding plasmids leads to the expression from the first ATG, and the resulting isoform has a restricted cytoplasmic distribution (Claudiani *et al*., 2005). In the cytoplasm, spastin is plentiful in the spindle pole and midbody, and the distal portion of

axons of immortalized motor neurons (Errico *et al*., 2004). Furthermore, *Drosophila* homologue of spastin, D-spastin was shown to be abundant at the axons and synaptic areas (Trotta *et al*., 2004). Nuclear role of spastin has not been clarified yet.

Spastin is essential for microtubule (MT) integrity. Like p60 katanin, another member of the AAA protein family, spastin can bind and disassemble microtubules in an ATPase dependent manner (Azim *et al.*, 2000; Errico *et al.,* 2002; Evans *et al*., 2005; Roll-Mecak and Vale, 2005). However, spastin does not include a mitochondrial signal peptide and examination of muscle biopsies from SPG4 patients did not reveal any mitochondrial abnormalities (Hazan *et al*., 1999; Hedera *et al*., 2000).

Importance of spastin for MT integrity was verified *in vivo* using its *Drosophila* orthologue. RNAi knockdown of D-spastin in *Drosophila* resulted in abnormal stabilization of microtubule cytoskeleton in neurons with synaptic growth and transmission deficiency. Employment of two pharmacological agents nocodazole and taxol, ameliorated these anomalies by modifying MT stability (Trotta *et al*., 2004).

Until now, more than 150 SPG4 variations have been reported including missense, splite-site, nonsense, deletion and insertion mutations. These mutations affect all coding regions of the gene, except exon four, and produce premature termination codons leading to decreased spastin mRNA levels at different tissues of patients (Burger *et al.,* 2000). However, most of the missense mutations were located to the AAA domain showing the importance of this region. Large deletions resulting in skipping of several exons were also determined (Burger *et al*., 2000; Sauter *et al*., 2002; Depienne *et al.,* 2007). These results support that haplo-insufficiency or loss-of-function may be the molecular cause of the disease. Furthermore, some N-terminal missense alterations such as p.S44L, p.E43Q and p.P45Q are suggested to affect the severity of the disease and cause anticipation in at least one affected family (Fonknechten *et al.,* 2000; Schickel *et al*., 2007). However, a proper genotype-phenotype correlation needs more investigation for SPG4.

1.1.3. SPG6

Mutations in NIPA1 gene are responsible for autosomal dominant spastic paraplegia 6 (SPG6). The disease specific locus was located on chromosome 15q11-q13 that is commonly deleted in Prader Willi (PWS) or Angelman Syndrome (AS) (Fink *et al*., 1995b). Genetic imprinting is the main indicator of these diseases, thus a large family with SPG6 was analyzed for the presence of genetic imprinting at the locus. However, no evidence could be identified for methylation status variation (Fink *et al.,* 1995a). Later, the same family was screened for mutations in four candidate genes including *NIPA1, NIPA2, GCP5, CYFIP1* that were highly conserved, nonimprinted and located closely to the imprinted domain and *NIPA1* was found to be mutated (Rainier *et al*., 2003).

Until now, four different mutations were reported in *NIPA1*. The first mutation was a nucleotide substitution at position 159 (T45R) and was identified in two unrelated Irish and Iraqi families (Rainier *et al*., 2003). Two other novel mutations (c.316G>C and c.316G>A) affecting the third exon of the gene were identified in two unrelated Chinese families and predicted to impair the third transmembrane domain of the protein (Chen *et al*., 2005). Later, these mutations were identified in other affected families from different geographic regions (Reed *et al*., 2005; Munhoz *et al*., 2006; Klebe *et al*., 2007; Liu *et al*., 2008). The forth one was a c.298G>A mutation in the third exon of the gene that was identified in a Japanese family (Kaneko *et al*., 2006).

NIPA1 consists of five exons and shares no homology with other HSP causing genes, but it shows significant homology with *NIPA2*. *NIPA1* is known to encode for nine transmembrane domains (TMDs) suggesting that it may be a member of TMD domain superfamily (Chai *et al*., 2003). Recently, *NIPA1* was shown to be important for the regulation of cellular magnesium homeostasis and several mutations of the gene were reported to change intracellular trafficking of NIPA1 protein and disrupt Mg^{+2} transport (Goytain *et al*., 2007).

1.1.4. SPG31

The SPG31 has been localized to chromosome 2q12 by genome wide screening in two large Caucasian families (Züchner *et al*., 2006a). The candidate interval was narrowed to approximately 8.8 Mb region between markers D2S139 and D9S2181 (Züchner *et al*., 2006a). After screening of nine candidate genes in this interval, six different mutations were identified in receptor enhancing-expression protein 1 gene (*REEP1*), also known as '*C2orf23*'. In this study, one missense mutation (c.59C→A; Ala20Glu), one deletion (c.526delG; Gly176fs), a single base pair deletion (c.507delC), a splice-site mutation (c.182-2A→G), and two 3'UTR alterations (c.606+43G→T and c.606+506 G→A) were defined. Mutations at 3'UTR site alter the sequence of a predicted conserved binding site of the miRNA gene; *miR-140*. These alterations result in reduced levels of REEP1 protein by promoting suppressive miRNA mediated effects on translation (Züchner *et al*., 2006b).

Züchner (2006b) suggested that REEP1 was a mitochondrial membrane protein since it has two predicted transmembrane domains and endogenous REEP1 was shown to localize only to mitochondria in COS-7 and MN-1 cell lines (Züchner *et al*., 2006b). Previously, it has been localized to Golgi and was suggested to have a role in secretory pathway (Saito *et al*., 2004).

Beetz *et al*. (2008) identified 13 novel and two previously identified mutations in all exons, except exon three, in a multi-national study comprising 535 unrelated HSP patients (Figure 1.2). Among those, splice site mutations, small insertions and deletions, and missense mutations were detected by conventional PCR and direct sequencing techniques. The group also identified a large multi exonic *REEP1* duplication was in one affected individual by multiplex ligation-dependent probe amplification (MLPA) assay (Beetz *et al.*, 2008).

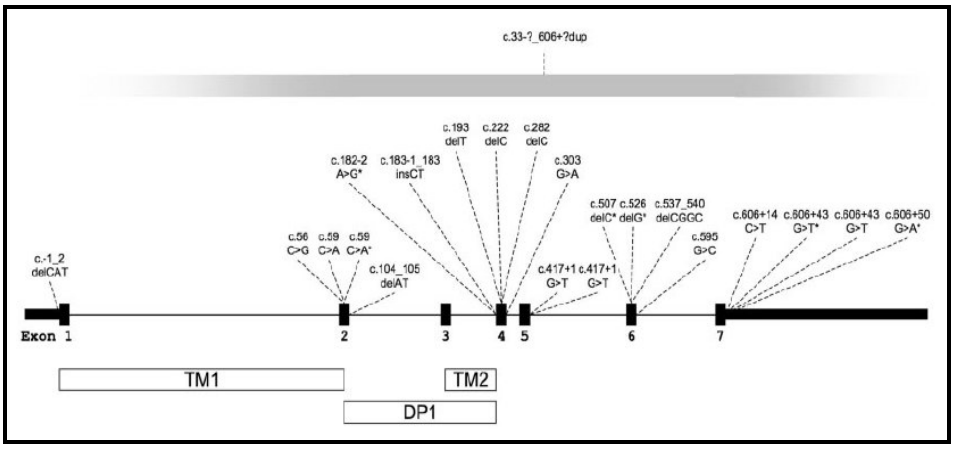


Figure 1.2. Distribution of mutations on the representative *REEP1* gene (Beetz *et al*., 2008).

1.1.5. SPG8

Age of onset of SPG8 is the second or third decade and it is accepted as one of the most severe forms of HSP (Hedera *et al.,* 1999). A muscle biopsy study was done with two SPG8 affected family members and remarkable histological or histochemical changes were not detected (Hedera *et al*., 1999; Rocco *et al*., 2000).

Linkage analysis in a Caucasian family has led to map the SPG8 locus to 8q23-24 region (Hedera *et al.*, 1999) and a following study narrowed the candidate region from 6.2 cM to 3.4 cM (Reid *et al*., 1999a). Several genes spanning the candidate SPG8 locus were screened among different families and three point mutations were detected in exons 11, 14, and 15 of the KIAA0196 gene. This gene consists of 28 exons and produces an 1159 aa protein called strumpellin. It is expressed ubiquitously and without alternative splicing (Valdmanis *et al*., 2007).

The first mutation, p.V626F, was identified in three large North American families with European ancestry and in one British family. The second mutation, p.L619F, defined in a Brazilian family was very close to the previous one at nucleotide sequence. Affected amino acids at positions 619 and 626, and the surrounding region were strictly conserved among all examined species suggesting that instead of mutated amino acids,

the region as a whole may be important for the protein function. The third mutation, p.N471D, was defined in a North American family with European ancestry. Position 471 is also conserved between all species except *D. melanogaster* and *X. tropicalis* (Valdmanis *et al*., 2007).

The strumpellin protein is predicted to have a spectrin repeat containing domain between amino acids of 434-518, and an α-helix structure between residues 606-644. In this regard, mutation at position of 471 may prevent the interaction of this predicted domain with other spectrin-repeat containing proteins. On the other hand, mutations p.V626F and p.L619F were suggested to impair the stability of α-helix structure (Valdmanis *et al*., 2007).

The exact function of the protein is still not known but based on its single spectrin repeat, strumpellin is suggested to interact with one of the cytoskeleton repeats. Therefore, it may have a role in signal transduction or protein localization processes in the cell (Valdmanis *et al*., 2007).

1.1.6. SPG10

The SPG10 disease severity is variable; some patients become wheelchair-dependent and yet others may not show symptoms until their adulthood. SPG10 locus was mapped to chromosome 12q13 by linkage analysis in a British family (Reid *et al*., 1999b; Reid *et al*., 2001). The heterozygous N256S mutation in the neuronal specific kinesin gene, *KIF5A*, was defined in all affected members of the same British family. The mutation appears in a highly conserved asparagine residue in the switch-two-helix motif of the motor domain that is essential for the functional kinesin (Reid *et al*., 2002). It was shown that mutations in this residue in orthologues KIF proteins resulted in interference of motor ATPase stimulation by microtubule binding (Soderblom and Blackstone, 2006). The second mutation 838C>T (R280C), was reported in the same motif required for the interaction with microtubules (Fichera *et al*., 2004).

Based on the fact that SPG10 is segregating as an autosomal dominant disease, kinesin mutations are thought to cause a dominant negative disease phenotype. Mutant

kinesins are suggested to interact with wild type ones and disrupt plus-end-directed microtubule motor function, thus they probably impair anterograde transport of membranous organelles in axons. Consistently, kinesin mutants in *Drosophila* lead to motor neuron disease phenotypes by causing the accumuation of organelles that disrupt anterograde and retrograde axonal transport (Hurd and Saxon, 1996).

An evidence was provided for the association between KIF5A and neurofilament transport (Xia *et al*., 2003). Postnatal inactivation of *KIF5A* expression in mice resulted in aggregation of neurofilament subunits in neuronal cell bodies, and reduced axon size leading to an age dependent degeneration of sensory neurons (Xia *et al*., 2003).

1.1.7. SPG13

SPG13 was mapped to chromosome 2q24-34 in a French family (Fontaine *et al*., 2000). The genes encoding the human mitochondrial chaperonin Hsp60 (heatshock protein 60; Cpn60) and its co-chaperonin Hsp10 (heat-shock protein 10; Cpn10) had been already located to this region, so they were accepted as candidates for SPG13. After mutation screening, a heterozygous G→A variation at position 292 (p.V98I) and a c.551A→G (p.N158S) mutation were identified in *HSP60* (Hansen *et al*., 2002).

GroEL is the *Escherichia coli* orthologue of *HSP60* and is important for bacterial growth (Fayet *et al*., 1989). Further studies with *Escherichia coli* strains lacking endogeneous orthologs showed that expression of the wild-type human *HSP60-HSP10* and p.N158S mutant constructs supported the growth of bacteria, whereas p.V98I mutant *HSP60-HSP10* constructs did not promote bacterial growth, implying the importance of this residue for proper protein function (Hansen *et al.*, 2002).

Haploinsufficiency of the functional Hsp60 may be the reason of autosomal dominantly inherited SPG13. On the other hand, mutant Hsp60 chaperonin subunits may show a dominant negative effect by forming complexes consisting of both active wild type and functionally insufficient Hsp60 (V72I) subunits that is expected to reduce the overall chaperonin activity. This is particularly important under stress conditions because

up-regulation of wild type allele would not be enough for the compensation of mutant allele. (Hansen *et al*. 2002; Bross *et al*., 2008).

1.1.8. SPG17

SPG17 is a rare type of HSP and the main signs are spasticity, weakness and amyotrophy of hand and feet muscles. A genome wide linkage study in an Austrain family assigned the locus to chromosome 11q12-q14. The candidate interval was reduced to approximately 13 cM region spanning between markers D11S1765 and D11S4136 (Patel *et al.,* 2001). Two heterozygous missense mutations, N88S and S90L, were identified in *BSCL2* that were suggested to impair conserved N-linked glycosylation consensus site (Windpassinger *et al*., 2004). *BSCL2* encodes seipin, a predicted integral membrane protein and mutated in another disease, autosomal recessive Berardinelli-Seip congenital lipodystrophy (CGL) type 2 (Agarwal and Garg, 2004).

The clinical features and the protein products of these eight loci and the other autosomal dominant HSP loci are summarised in Table 1.1.

Table 1.1. Autosomal dominant forms of HSP (modified from Depienne *et al.*, 2007).

Type	Locus	Chromosome region	Gene or Product	Discriminating features
Pure	SPG3A	14q12-q21	Atlastin	Predominantly early onset
	SPG4	2p22	Spastin	-
	SPG6	15q11.2-q12	NIPA1	Predominantly adult onset
	SPG8	8q24	KIAA0196	Predominantly adult onset
	SPG10	12q13	KIF5A	Predominantly early onset
	SPG12	19q13	Unknown	Predominantly early onset
	SPG13	2q24-q34	HSP60	Predominantly adult onset
	SPG19	9q33-q34	Unknown	Predominantly adult onset
	SPG31	2p12	REEP1	-
	SPG33	10q24.2	ZFYVE27	-
	SPG37	8p21.1-q13.3	Unknown	-

Table 1.1. Autosomal dominant forms of HSP (modified from Depienne *et al.*, 2007) (continued).

Complex	SAX1	12p13	Unknown	Spastic ataxia
	SPG9	10q23.3q24.2	Unknown	Cataract, motor neuropathy, skeletal abnormalities, reflux, short stature
	SPG17	11q12-q14	BSCL2/ Seipin	Silver syndrome; severe distal wasting
	SPG29	1p31-p21	BSCL2/ Seipin	pes cavus, disrupted hearing, hiatal hernia, neonatal hperbilirubinemia without kernicterus

1.2. Autosomal Recessive HSP

Autosomal recessive HSPs are less common than autosomal dominant types. Both pure and complicated forms of the disease have been observed.

1.2.1. SPG7

SPG7 may appear either as pure or complicated and complicated form has additional features such as cerebellar and cortical atrophy, optic atrophy or peripheral neuropathy. The locus responsible for SPG7 was mapped to chromosome 16q24.3 (De Michele *et al*., 1998) and disease causing mutations were identified in the paraplegin gene (Casari *et al*., 1998). Paraplegin is the mammalian orthologue of the yeast m-AAA protease and belongs to the mitochondrial metalloproteases, a subgroup of the AAA protein family (Casari *et al.*, 1998). Similar to other members of this family, paraplegin has an AAA domain including Walker A and B motifs (Reid, 2003a). Localization studies showed that paraplegin is abundant in the subcellular compartments of mitochondria (Casari *et al*., 1998).

A paraplegin related gene, *AFG3L2*, was identified and mapped to chromosome 18p11 (Banfi *et al*., 1999). Paraplegin and AFG3L2 compose a large complex in the inner membrane of mitochondria (Atorino *et al*., 2003). This complex has a reduced mitochondrial complex I activity and is more susceptible to oxidative stress in SPG7 patients. However, overexpression of wild-type *paraplegin* overcomes these abnormalities in several cell lines from HSP patients. Accordingly, studies using muscle biopsies of patients showed the presence of ragged red fibers, cytochrome c oxidase deficient fibers or abnormal accumulation of mitochodria suggesting that paraplegin mutations impair the mitochondrial function (Casari *et al*., 1998; McDermott *et al.,* 2001). Moreover, a study with paraplegin null mouse models showed abnormal mitochondria aggregation at axons which in turn leads to axon swelling due to accumulated organelles and neurofilaments. These results imply that mitochondrial impairments may cause axonal degeneration by damaging both anterograde and retrograde axonal transport (Ferreirinha *et al.,* 2004).

1.2.2. SPG11

SPG11 is a common form of ARHSP with thin corpus callosum and may begin at either childhood or adolescence. Main feature of the disease is the fast progress of the lower limb spasticity which leads to wheelchair-dependence at ages 30-40. Progressive cognitive impairment and mild or moderate mental retardation are the other common features of the disease (Depienne *et al*., 2007).

The SPG11 locus was mapped to chromosome 15q13-q15 by linkage analysis in Japanese families. Patients in these families had mental retardation and thin corpus callosum instead of spasticity (Shibasaki *et al*., 2000). Four nonsense, four small deletion and two small insertion mutations were identified in these patients in *SPG11/KIAA1840* proving that *SPG11* is the causative gene. All of these mutations result in the generation of premature stop codons supporting that the disease may appear as a result of loss of function mechanism (Stevanin *et al*., 2007).

The SPG11 gene consists of 40 exons and encodes a protein spatacsin, with 2443 amino acids and an unknown function. The sequence of spatacsin is highly conserved between orthologs in mammals and other vertebrates (Stevanin *et al.*, 2007).

1.2.3. SPG20

Troyer Syndrome (SPG20) is a complicated type of HSP and common in Amish population. The principal characteristics of the disease are spastic paraparesis with distal amyotrophy, dysarthria, and delayed growth (Cross and McKusick, 1967). The SPG20 gene consists of nine exons and encodes spartin with 666 amino-acid residues. Patel *et al*. (2002) identified a 1110delA frameshift mutation in the exon four of *SPG20* leading to 29 amino acid long substitution. Localization studies showed that spartin is ubiquitously expressed, but is abundant in adipose tissue. Spartin has a partial sequence homology with several proteins including SNX15, VPS4, Skd1 and the amino-terminal region of spastin (Patel *et al.*,2002).

The clinical features and the protein products of these three loci and the other autosomal recessive HSP loci are summarized in Table 1.2.

Table 1.2. Autosomal recessive forms of HSP (modified from Depienne *et al*., 2007).

Type	Locus	Chromosome region	Gene or Product	Discriminating features
Pure	SPG5	8p	Unknown	-
	SPG24	13q	Unknown	-
	SPG28	14q	Unknown	-
	SPG30	2q	Unknown	-
Complex	SPG7	16q	Paraplegin	Cerebellar involvement, PNS, pes cavus, optic atrophy
	SPG14	3q	Unknown	Distal motor neuropathy, mental retardation, pes cavus, visual agnosia

Table 1.2. Autosomal recessive forms of HSP (modified from Depienne *et al*., 2007) (continued).

Complex	SPG27	10q	Unknown	Cerebellar ataxia, PNP, mental retardation, microcephaly, facial and skeletal dysmorphia
	SPG11	15q	Spatacsin	Mental retardation or cognitive impairment, PNP, TCC
	SPG15	14q	Unknown	Pigmented maculopathy, wasting, dysarthria, cerebellar signs, mental retardation
	SPG20	13q	Spartin	Mental retardation, cerebellar signs, developmental delay, short stature
	SPG21	15q	Maspardin	Cerebellar signs, premature aging, cognitive decline, extrapyramidal syndrome, dysarthria, TCC, cataract
	SPG23	1q	Unknown	Hair and skin pigmentation abnormalities, facial and skeletal dysmorphia, postural tremor, cognitive impairment
	SPG25	6q	Unknown	Bilateral cataract, congenital glaucoma, multiple disc herniation

Table 1.2. Autosomal recessive forms of HSP (modified from Depienne *et al*., 2007) (continued).

Complex	SPG26	12p11.1-q14	Unknown	Intellectual impairment, distal muscle wasting, dysarthria, PNP
	SPG32	14q	Unknown	Mental retardation, TCC
	TCC+ epilepsy	8q	Unknown	Mental retardation, TCC, epilepsy
	SPOAN	11q	Unknown	Optic atrophy, PNP
	ARSACS	13q	Sacsin	Ataxia, dysarthria, distal wasting, nystagmus, retinal striation, PNP
	ARSAL	2q	Unknown	Spastic ataxia, leucodystrophy
	SAX2	17p	Unknown	Cerebellar ataxia, dysarthria

1.3. X-Linked HSP

Until now, three different loci were found to be responsible for X-linked HSP; SPG1, SPG2 and SPG16. Mutations of neuronal cell adhesion molecule L1 (*L1CAM*) gene were reported to cause SPG1, whereas proteolipid protein (*PLP1*) gene mutations lead to SPG2. While SPG1 only shows complicated pattern of HSP, SPG2 may be both complicated or pure (Jouet *et al.*, 1994; Saugier-Veber *et al.*, 1994). The gene responsible for SPG16 has not been identified yet.

1.3.1. SPG1

SPG1 locus was mapped on Xq28 and mutations in the L1CAM gene in this locus were identified in patients (Jouet *et al.*, 1994). These mutations result in pure HSP, more commonly, in combination with two other complex neurological disorders and are frequently named as the “CRASH syndrome” (corpus callosum hypoplasia, retardation,

adducted thumbs, spastic paraplegia, and hydrocephalus) or “MASA syndrome” (mental retardation, adducted thumbs, shuffling gait and aphasia) (Fransen *et al.*, 1996).

The disease associated with *LICAM* mutations starts in the first two decades of life and the symptom progression is slow. Besides, affected individuals acquire motor functions by time. For these reasons, it was suppossed that the abnormal development of the corticospinal tract may cause the disease. Consistently, neuropathological studies showed that the pyramidal cells are absent or their size is decreased remarkably in SPG1 patients (Yamasaki *et al.*, 1995). Furthermore, weakness of hind limbs and reduced size of corticospinal tracts were detected in transgenic animals with impaired L1CAM gene emphasizing the requirement of L1CAM molecule for normal development of the corticospinal tracts (Casari and Rugarli, 2001).

The L1CAM protein is a cell surface transmembrane glycoprotein which belongs to immunoglobulin (Ig) superfamily. It is expressed mainly in the nervous system, specifically on the surface of long axons and growth cones during development (Joosten and Gribnau, 1998). The L1CAM protein binds to several transmembrane proteins (such as L1 CAM molecules), extracellular matrix proteins, several extracellular ligands (such as other CAMs, integrins, proteoglycans), and intracellular proteins (such as ankyrin) in order to activate signal transduction pathways (Brummendorf and Rathjen, 1996; Burden-Gulley *et al.*, 1997; Reid, 2003a). It has roles in various cellular processes such as axon pathfinding, axon and dendrite projections, and cell adhesion (Casari and Rugarli, 2001).

To date, many mutations were identified in SPG1 patients. These mutations are very heterogeneous and affect nearly all identified functional domains (Fransen *et al.*, 1997; Weller and Gärtner, 2001).

1.3.2. SPG2

PLP1 encodes proteolipid protein 1 and its splicing variant DMD20. PLP1 is one of the essential protein components of myelin in the central nervous system (Crosby and Proukakis, 2002; Soderblom and Blackstone, 2006).

Mutations in *PLP1* can cause Pelizaeus–Merzbacher disease (PMD) or both pure and complicated HSP (SPG2). According to the mutation type, symptoms can begin at any age and severity of the disease can be apparently different among patients. Main features of PMD are hypomyelination of the CNS and decrease in size of mature oligodendrocytes. PMD is more severe than SPG2; it may begin at infancy and cause death in childhood. In contrast, SPG2 generally begins in adult stage and has a milder nature (Soderblom and Blackstone, 2006).

It is suggested that interruption of the axon-neuroglia interactions and the resulting transport abnormalities due to PLP1 deficiency may cause axonal degeneration observed in SPG2 patients (Yool *et al*., 2000; Casari and Rugarli, 2001).

2. AIM OF THE STUDY

In the scope of this study, we aimed to investigate the molecular basis of HSP in a cohort of 42 unrelated Turkish HSP families and CMT patients with lower limb spasticity. Up to date, 39 loci and 17 genes have been reported to cause HSP. Among them, *Atlastin*, *Spastin*, *NIPA1* and *REEP1* are the most commonly mutated genes and are known to be responsible for 50 per cent of autosomal dominant HSP cases. We aimed to initiate the first study of autosomal dominant HSP in the Turkish population by investigating the above mentioned genes. Although these genes are known to be cause pure forms of the disease, to unravel the contribution of these genes to the complicated HSP phenotype we have investigated patients presenting with both pure and complicated forms.

Clinical evaluation is very important and genetic studies should be a part of clinical analysis for differential diagnosis of HSP. For this reason, we also aimed to perform genotype/phenotype correlation for the mutated cases to further our understanding about the nature of the disease and to unravel the disease mechanism.

3. MATERIALS

3.1. Subjects

Peripheral blood samples of Turkish HSP patients and their family members were provided by the following neurology departments: Department of Neurology, Istanbul Medical Faculty and Cerrahpasa Medical Faculty, Istanbul University; Department of Neurology, Pamukkale University; Department of Neurology, Marmara University; Department of Neurology, Gazi University.

3.2. Chemicals

All solid and liquid chemicals used in this study were purchased from Merck (Germany), Sigma (USA), Riedel de-Häen (Germany) and Carlo Erba (Germany), unless stated otherwise in the text.

3.3. Buffers and Solutions

3.3.1. DNA Extraction from Peripheral Blood

Cell Lysis Buffer	:	155 mM NH_4Cl 10 mM $KHCO_3$ 1 mM Na_2EDTA (pH 7.4)
Nuclease Lysis Buffer	:	10 mM Tris-HCl (pH 8.0) 400 mM NaCl 2 mM Na_2EDTA (pH 7.4)
Sodiumdodecylsulphate (SDS)	:	10 per cent SDS (w/v) (pH 7.2)
Proteinase K	:	20 mg/ml in H_2O

5 M Sodium NaCl Solution	:	292.2 g NaCl in 1 L dH_2O
Ethanol (EtOH)	:	Absolute EtOH
Tris-EDTA (TE) Buffer	:	20 mM Tris-HCl (pH 8.0) 1 mM Na_2EDTA (pH8.0)

3.3.2. Polymerase Chain Reaction (PCR)

Magnesium Chloride ($MgCl_2$)	:	25 mM $MgCl_2$ (Fermentas, Lithuania)
10 X $MgCl_2$ Free Buffer	:	100 mM Tris-HCl 500 mM KCl (Fermentas, Lithuania)
Deoxyribonucleotide Triphosphates (dNTPs)	:	100 mM of each dNTP (Fermentas, Lithuania)
Dimethylsulphoxide (DMSO)	:	Stock solution (Fermentas, Lithuania)

3.3.3. Agarose Gel Electrophoresis

10 X Tris-Boric Acid-EDTA (TBE) Buffer	:	0.89 M Tris-Base 0.89 M Boric acid 20 mM Na_2EDTA (pH 8.3)
Ethidium Bromide (EtBr)	:	10 mg/ml
1 or 2 per cent Agarose Gel	:	1 or 2 per cent (w/v) Agarose in 0.5X TBE Buffer

10 X Loading Buffer	:	2.5 mg/ml Bromophenol Blue (BPB) 1 per cent SDS in 2 ml glycerol
DNA Ladder	:	100 bp, MBI (Frementas, Lithuania)

3.3.4. Polyacrylamide Gel Electrophoresis

10 X TBE Buffer	:	0.89 M Tris-Base 0.89 M Boric Acid 20 mM Na_2EDTA (pH 8.3)
30 per cent Acrylamide Stock (29:1)	:	29 per cent Acrylamide 1 per cent N, N'-methylenebisacrylamide
Ammonium Persulfate (APS)	:	10 per cent APS (w/v)
10 X Denaturing Buffer	:	95 per cent formamide 20 mM EDTA 0.05 per cent Xylene Cyanol 0.05 per cent Bromophenol Blue

3.3.5. Silver Staining

Buffer A	:	10 per cent Ethanol 0.5 per cent Glacial Acetic Acid
Buffer B	:	0.1 per cent $AgNO_3$ in dH_2O
Buffer C	:	1.5 per cent NaOH 0.015 per cent Formaldehyde
Buffer D	:	0.75 per cent Na_2CO_3

3.4. Fine Chemicals

3.4.1. Enzymes

Taq DNA Polymerase, the restriction enzymes HpaII (MspI) and NIaIV (BspLI) were purchased from Fermentas (Lithuania). The restriction enzyme PstI was obtained from Roche (USA).

3.4.2. Oligonucleotide Primers

The sequences of the primers to amplify exons of *Atlastin, Spastin*, *NIPA1*, and *REEP1* are given in Tables 3.1, 3.2, 3.3 and 3.4, respectively. These primers were purchased from Iontek (Turkey).

Table 3.1. Sequences of the primers used for exon amplification of *Atlastin*.

Exon	Primer (F/R*)	Primer Sequence (5'→3')	Size (bp)	Annealing Temp.(°C)
Exon 1	SPG3A 1F	GAGGGTGTGACGCTGGTATC	328	63
	SPG3A 1R	AAGTGGAGGGCCAGAAGACC		
Exon 2	SPG3A 2F	CTGTGTCGGATGTTTGAGAG	487	63
	SPG3A 2R	TGGAATGGTTACACCACAGC		
Exon 3	SPG3A 3F	TCGAATTGGAGAGGGATAAG	308	62
	SPG3A 3R	AAGTGCAACTTCAAGGATCC		
Exon 4	SPG3A 4F	TGGTAACCCTAATGACCTAG	274	61
	SPG3A 4R	ATGATTCCCAATTTCTGTTG		
Exon 5	SPG3A 5F	GTAGGGAATGATGAAGTAAG	257	61.5
	SPG3A 5R	CTAATTGGGCCAATAGTTCC		
Exon 6	SPG3A 6F	GTTATACCTAGAGGGAAAAG	255	59
	SPG3A 6R	GACCCTAATTAATATACCTGG		
Exon 7	SPG3A 7F	GGCACCTTAAAGTCCTCATA	208	53
	SPG3A 7R	CACCAAATGATCCAACAGA		

* F: Forward primer, R: Reverse primer

Table 3.1. Sequences of the primers used for exon amplification of *Atlastin* (continued).

Exon 8	SPG3A 8F	TTAGTAGCAGCCCTGTCGTG	384	54.1
	SPG3A 8R	CATCAGCCTCCTATCAGTGG		
Exon 9	SPG3A 9F	TGGAGGACTGGGAAGGATTC	293	63
	SPG3A 9R	TTCCTCGTACCTTTGCTCCC		
Exon 10	SPG3A 10F	GCATTTCAGGAAAGGGAAAC	310	63
	SPG3A 10R	ATTTCTGACAGCCAGAAATC		
Exon 11	SPG3A 11F	GAAATGTGAACTGCCTGTGG	204	63
	SPG3A 11R	AGTTGCATGAAGGATACTGG		
Exon 12	SPG3A 12F	GCAGGCTCCTGATTATTAAC	624	54.1
	SPG3A 12R	TCTAATGCAGTGGCTGGCAC		
Exon 13	SPG3A 13F	CTGCAGGAGTATCTGTTCTG	253	53
	SPG3A 13R	CACCAAAGATTGTTCTAATC		
Exon 14	SPG3A 14F	ATGCACACATTGAGGAGTTG	392	63
	SPG3A 14R	TACTCCGTTCTGATGGAAGC		

Table 3.2. Sequences of the primers used for exon amplification of *Spastin.*

Exon	Primer (F/R*)	Primer Sequence (5'→3')	Size (bp)	Annealing Temp. (°C)
Exon 1	SPAST 1aF	TACTATTTCTCCTACCCGCTGTT	321	63-43
	SPAST 1aR	CGACCCCACCGCCTTCT		
	SPAST 1bF	CGGCGGCGGCAGTGAGAG	284	63-43
	SPAST 1bR	ATGAGGGCGCGGGAGAAG		
Exon 2	SPAST 2F	TTTTTATGTATTACCTCTCAAC	204	57
	SPAST 2R	TGGGATGGCTATAAACAAAT		
Exon 3	SPAST 3F	TTAGTTGGGAAATGTAGAT	246	56.5
	SPAST 3R	TATGTTAAAAAGCCTGGAC		
Exon 4	SPAST 4F	TTTTTACCTTCTCTGTTG	156	55
	SPAST 4R	AAGCTTTATTATTTTATGTTAGT		
Exon 5	SPAST 5F	GTTCAGCTACAATTTTCTAATCC	321	54.5
	SPAST 5R	TATGATCAACTTAAGCAGGAAT		
Exon 6	SPAST 6F	ATGTTAGGTTGTATTTTCA	273	56
	SPAST 6R	CAAGGTATTTATTATCTATTTCC		

* F: Forward primer, R: Reverse primer

Table 3.2. Sequences of the primers used for exon amplification of *Spastin* (continued).

Exon 7	SPAST 7F	TCATAGGGCTTAGGCTTCA	224	62.5
	SPAST 7R	ATGGATTCAGTAACAGATGGTAT		
Exon 8	SPAST 8F	CTGTTTGGGAAGATGCT	261	58.5
	SPAST 8R	CTCAAGGACAAGATAAAGTT		
Exon 9	SPAST 9F	TGGCCTCATAGCTTACATTTTTAG	219	63
	SPAST 9R	TACGACAATATTGGAAACAGAG		
Exon 10	SPAST 10F	GTGCTAGATTTTCAACATA	270	54
	SPAST 10R	GCCCTTCTTTAAAACTTCTTCC		
Exon 11	SPAST 11F	GAATTTAGTAGGACCCACT	230	53
	SPAST 11R	GCCACATTAAAAATATCATA		
Exon 12	SPAST 12F	ATGGCCAAGGTTAAAAATACAA	281	54
	SPAST 12R	CTGGAAGAAAATAGTGAAT		
Exon 13	SPAST 13F	CTTTTCCTGTCATTTGCTGTTT	173	53
	SPAST 13R	GATGGTAGTTCTTGTTTCTGCTCT		
Exon 14	SPAST 14F	ATCATTAATTCTGAAATTAG	154	50
	SPAST 14R	ATAAACCAAATCCAAA		
Exon 15	SPAST 15F	AAAAAGCGGGAGGGGAAATA	248	53
	SPAST 15R	TGGGCAACAGAGTGAGACC		
Exon 16	SPAST 16F	TGTATGTATTTTTAAGTGCCTGAC	124	56
	SPAST 16R	TACAATATAGAAGACAAAGAAA		
Exon 17	SPAST 17F	AACAGCAGCATCATTACTTT	241	58
	SPAST 17R	GTTCTGCAGGTTTACAA		

Table 3.3. Sequences of the primers used for exon amplification of *NIPA1*.

Exon	Primer (F/R*)	Primer Sequence (5'→3')	Size (bp)	Annealing Temp.(°C)
Exon 1	NIPA1.1F	CTCTTCCTGCTCCTCCCCCA	373	50
	NIPA1.1R	CACCTGCGACCGCCTTCTC		
Exon 2	NIPA 2F	TTCTCGCTTGTAAACCTCTTCC	158	59.4
	NIPA 2R	AAGACCAGACTTCGCCACAC		
Exon 3	NIPA 3F	CAACTGTGATCAGTGCTGGA	180	61.3
	NIPA 3R	CAAACACAATCTTGGCTCTCAC		

* F: Forward primer, R: Reverse primer

Table 3.3. Sequences of the primers used for exon amplification of *NIPA1* (continued).

Exon 4	NIPA 4F	GAAGAAAGGTCAGGTAGTTTGG	242	62.5
	NIPA 4R	GCAGTGCTGCTAGAAAGGAA		
Exon 5	NIPA 5F	TAAATTCAAGCCAGAGCCCA	706	63.4
	NIPA 5R	TGCCTATTCCTCGAACCATC		

Table 3.4. Sequences of the primers used for exon amplification of *REEP1*.

Exon	Primer (F/R*)	Primer Sequence (5'→3')	Size (bp)	Annealing Temp.(°C)
Exon 1	REEP1.1F	GCTGACTGACGTTCCGGG	145	63
	REEP1.1R	GGGGGAGAAGGCCACTTA		
Exon 2	REEP1.2F	TTTTTGTCTCTCTTCCCTCCA	127	61.3
	REEP1.2R	CAACCCGCTCGAAAATACAC		
Exon 3	REEP1.3F	GGTGTTGGGCAGTGTTTTTC	241	61.3
	REEP1.3R	CTGAGGTGAGGTTTGAGGCT		
Exon 4	REEP1.4F	GCAGTGTTGCTGAGAACCTAA	200	63.5
	REEP1.4R	GAATGAAAGACATGGCAGCA		
Exon 5	REEP1.5F	TGTGCCTCTGTTTTTCCTTTG	228	61
	REEP1.5R	TAGCCTGTTCTGTGTGGTCG		
Exon 6	REEP1.6F	GTGCCCCTCCTGTGTTGTAT	240	63.5
	REEP1.6R	CAAGGAGTGGGAAAGAGGG		
Exon 7	REEP1.7F	GCTGACCTCAAAAGATCCCA	208	59.4
	REEP1.7R	TTCCTGAAGCGAGATCGAAG		

* F: Forward primer, R: Reverse primer

3.4.3. DNA Size Markers

The size marker used in this study was 100-bp DNA ladder with a range of 100-1000 bp (Fermentas, Lithuania).

3.4.4. Other Fine Chemicals

QIAquick PCR Purification Kit was purchased from (Qiagen, Germany).

3.5. Equipment

Autoclaves	:	ASB270NT (Astell, UK)
Balances	:	Electronic Balance Model VA124 (Gec Avery, UK) Electronic Balance Model CC081 (Gec Avery, UK)
Centrifuges	:	Centrifuge 5415C (Eppendorf, Germany) Universal 16R (Hettich, Germany) Allegra X-22R Centrifuge (Beckman Coulter, USA)
Deep Freezers	:	-20 ºC (Bosch, Germany) -20 ºC 2021D (Arçelik, Turkey) -70 ºC (GFL, Germany)
Documentation System	:	BioDoc Video Documentation System (Biometra, Germany)
Electrophoretic Equipments	:	Horizon 58, Model 200 (BRL, USA) Sequi-Gen Sequencing Cell (Bio-Rad, USA)
Heat Blocks	:	Hotplate SH1D (Cytocell, UK)
Incubators	:	Shake'n'Stack (Hybaid, UK) Oven EN400 (Nüve, Turkey)
Magnetic Stirrer	:	Chiltern Hotplate Magnetic Stirrer HS3 (UK)

Ovens	:	Microwave Oven (Vestel, Turkey)
Power Supplies	:	Power Pac Model 3000 (Bio-Rad, USA) Apelex PS304 (Apelex, France) Standart Power Pac Model P25 (Biometra, Germany)
Refrigerator	:	2082C (Arçelik, Turkey)
Shaker	:	SL350 (Nüve, Turkey)
Spectrophotometer	:	CE 5502 Scanning Double Beam 5000 Series (CECIL Elegant Technology, UK) Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA)
Thermocyclers	:	iCycler (Bio-Rad, USA) MyCycler (Bio-Rad, USA) Techne (Progene, UK)
UV Transilluminator	:	Chromato-Vue Transilluminator Model 1-TM-20 UVP (USA)
Vortex	:	Nuvemix, NM110 (Nüve, Turkey)
Water Purification	:	WA-TECH Ultra Pure Water Purification System (WA-TECH, Germany)

4. METHODS

4.1. DNA Extraction from Peripheral Blood

Ten ml blood samples from individuals were taken into tubes containing K'EDTA that is necessary to prevent coagulation. The tubes were stored at +4 ºC until DNA extraction. As the first step of DNA extraction, the samples were taken into sterile Falcon centrifuge tubes. Thirty ml cold red blood cell (RBC) lysis buffer was added to the samples and kept at +4 ºC for 20 minutes in order to lyse the erythrocyte membranes. For the collection of the leukocyte nuclei, the samples were centrifuged at 5000 rpm for 10 minutes at +4 ºC. The supernatant was discarded and the nuclei were resuspended in 10 ml lysis buffer by vortexing. The samples were centrifuged again at 5000 rpm for 10 minutes at +4 ºC. After discarding the supernatant, the nuclear pellet was resuspended in three ml nuclei lysis buffer. Following the addition of 50 µl of 10 per cent SDS and 30 µl of Proteinase K, the samples were incubated at 37 ºC overnight or 56 ºC for three hours to reduce the amount of cellular proteins. Subsequently, 10 ml of five M NaCl was added to samples in order to salt out the protein debris and the samples were centrifuged at 5000 rpm at room temperature for 20 minutes. The supernatant was taken into a new sterile Falcon tube and two volumes of absolute EtOH were added for the precipitation of DNA. The DNA was fished out and taken into a sterile 1.5 ml Eppendorf tube. After the remaining ethanol was evaporated, Tris-EDTA (TE) buffer was added and the DNA was left overnight at room temperature in order to dissolve completely. Afterwards, it was stored at -20 ºC and the dilutions are kept at +4 ºC (Miller *et al*., 1988).

4.2. Quantitative Analysis of Extracted DNA

The concentration of the isolated genomic DNA was measured using Nanodrop ND-1000 spectrophotometer. In order to calculate the concentration, DNA-50 program was selected and optic density of extracted DNA was measured at 260 nm. The measurement was done according to the formula based on the fact that 50 µg of double stranded DNA has an absorbance of 1.0 at 260 nm.

4.3. Mutation Analysis of Atlastin, Spastin, NIPA1 and REEP1 Genes

Fourty two HSP patients were screened for probable sequence alterations in the exons of Atlastin (14q12-q21), Spastin (2p24-p21), Non-imprinted in Prader-Willi/ Angelman Syndrome 1 (NIPA1) (15q11.2-q12), and Receptor Expression Enhancing Protein 1 (REEP1) (2p12) genes using PCR, SSCP and subsequent DNA sequencing.

4.3.1. Polymerase Chain Reaction (PCR)

4.3.1.1. Primer Design. The coding exons and flanking intronic sequences of *Atlastin*, *Spastin*, *NIPA1*, and *REEP1* were amplified by PCR method. We used previously published primer sets for the amplication of *Atlastin* (Durr *et al*., 2004), and *Spastin* (Lindsey *et al*., 2000) given in Table 3.1. and 3.2. The other primers were designed according to *NIPA1* sequence (GenBank accession NT_078094) and *REEP1* sequence (NT_022184) using web-based software Primer3 (http://seqtool.sdsc.edu). The specifity of these primers were checked using the 'PCR' program at http://www.genome.ucsc.edu/. However, we could not overcome the nonspecific amplifications during the amplification of the first exon of *NIPA1*. Thus, we provided a new primer set for this exon from Dr. Andrew Crosby (UK).

4.3.1.2. PCR Conditions. PCR reactions were performed in a 25 µl volume containing approximately 100 ng genomic DNA, 1 X Mg^{2+}-free buffer, 2 or 2.5 mM of $MgCl_2$, 0.4 µM of each primer, 0.25 mM of each dNTP and 1 U of Taq DNA polymerase.

The PCR program used for the amplification of the exons of *Spastin* (except exon one) and *Atlastin* was as follows: an initial denaturation step at 95 °C for five minutes (min) followed by 35 cycles of 30 seconds (sec) at 95 °C, one minute at annealing temperature (Table 3.1 and 3.2), 30 sec at 72 °C, a final elongation at 72 °C for five min, and the cooling at 15 °C for one min. Primer sets SPAST 1a and 1b were used for the amplification of the first exon of *Spastin* by touchdown PCR; 40 cycles of touchdown

PCR consists of 45 sec at 95 °C, 45 sec at 63 °C with 0.5 °C decrement in each cycle, and 30 sec at 72 °C.

The PCR program used for the amplification of the exons of *NIPA1* (except exon one) and *REEP1* (except exon one) was as follows: an initial denaturation step at 95 °C for five min followed by 35 cycles of 45 sec at 95 °C, 45 sec at annealing temperature (Table 3.3 and 3.4), 50 sec at 72 °C, a final elongation at 72 °C for five min, and the cooling at 15 °C for one min. First exon of *NIPA1* was amplified using the same PCR procedure instead of extension time that was determined as 30 sec at 72 °C for this exon. On the other hand, *REEP1* exon one was amplified using touchdown PCR as follows: 40 cycles of 45 sec at 95 °C, 45 sec at 63 °C with 0.5 °C decrement in each cycle, and 20 sec at 72 °C.

PCR products were controlled by running the samples on two per cent agarose gel after PCR.

4.3.2. Single Strand Conformation Polymorphism (SSCP) Analysis

For the purpose of detecting differential migration patterns, the amplified products were run on an acrylamide gel with glycerol and on eight per cent acrylamide gel. The glass plates used for electrophoresis were 20 cm×20 cm and 22 cm×20 cm. The surfaces of the glass plates had to be very clean to hamper the occurrence of any interference. Thus, in order to get rid of any oily fingerprints and dust, the surfaces of the plates and one mm plastic spacers were wiped away using ethanol. The spacers were put on the two edges of the plates and clamps were used to stabilize and tighten the plates.

In order to prepare 60 ml eight per cent acrylamide gel, 16 ml of 30 per cent (29:1 acrylamide- bisacrylamide in dH_2O) stock acrylamide solution was mixed with 7.2 ml of 5 X TBE buffer. Total volume was adjusted to 60 ml with dH_2O. Afterwards, 600 µl of 10 per cent ammonium per sulfate (APS) and 60 µl of TEMED were added and the gel was poured into the area between the two plates. A plastic comb with 15 well was placed to the upper part of the gel and the gel was left for at least one hour at room temperature for polymerization.

The electrophoresis tank was filled with 0.6 X TBE buffer. After polymerization, the comb was removed, the gels were placed into the tank containing 0.6 X TBE buffer and the wells were cleaned using distilled H_2O. Each of the PCR product was mixed with the denaturing loading dye (95 per cent formamide, 0.05 per cent bromophenol blue, 0.05 per cent xylene cyanol) in 1:1 ratio. Before loading, the samples were denatured at 94˚C for five min and chilled on ice for five min. According to efficiency of PCR reaction, different amounts of the mix were loaded onto the wells. The gel was generally run for 20 hours at 90 Volts and the DNA fragments were visualized by silver-staining method.

4.3.3. Silver Staining

Silver-staining technique was performed to visualize the separated alleles on polyacrylamide gels. The gels were treated with Buffer A for three min to fix the DNA fragments. After removing Buffer A, the gels were incubated with one per cent silver nitrate solution, which is called Buffer B, for ten minutes. For the purpose of preventing non-specific staining, the gels were washed with distilled dH_2O after Buffer B. Afterwards, they were treated with freshly prepared Buffer C until the bands became visible. Buffer C was replaced with Buffer D, in which the gels were kept for five minutes to stop color reaction. Finally, the gels were taken into a transparent folder and all sides of the folder were sealed.

4.3.4. DNA Sequence Analysis

When a different migration pattern was detected, the patient's genomic DNA was amplified by PCR using the same primer pair. Then, PCR products were purified using QIAquick purification kit (Qiagen). Sequencing of both sense and antisense strands of the samples were performed using automated sequencer ABI 3700 PRISM (Applied Biosystems) by Iontek (Istanbul, Turkey).

4.3.5. Restriction Endonuclease Analysis

In order to confirm the presence of sequence variations, restriction endonuclease digestion was performed. For this aim, target exons were amplified using suitable primer sets. Ten µl of PCR product was mixed with 4 U of restriction enzyme and three µl of suitable buffer. Total volume was adjusted to 30 µl with dH_2O. The samples were kept at 37 °C overnight. The digestion products were run on two per cent agarose gel and stained using ethidium bromide to visualize under UV light.

5. RESULTS

For the purpose of understanding the molecular basis of autosomal dominant forms of HSP disease, forty two unrelated families were analyzed for mutations in the Spastin, Atlastin, NIPA1 and REEP1 genes. Thirty four of these families were clinically diagnosed as HSP whereas eight were referred to our laboratory for differential analysis CMT and HSP phenotypes. Nineteen of the families had a familial history, whereas other twenty two were sporadic cases.

5.1. SSCP Analysis

5.1.1. Variations in the Atlastin Gene

Single strand confirmation polymorphism was observed in exon eight of the Atlastin gene in patient H2 (Figure 5.1).

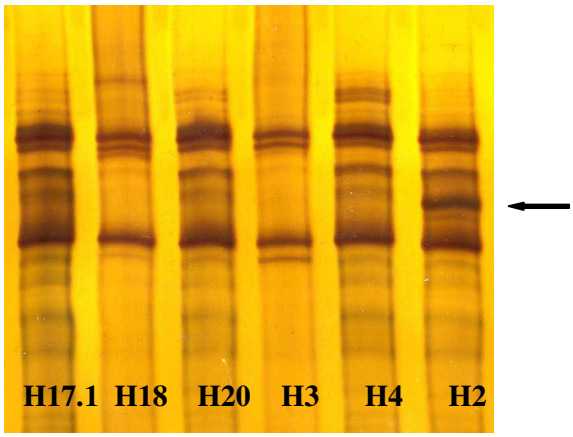


Figure 5.1. Eight per cent SSCP gel with glycerol indicating a different banding profile of exon eight of *Atlastin* in patient H2.

Sequencing analysis of patient H2 for exon eight of *Atlastin* revealed a heterozygous missense mutation, c.941A>G (p.H258R), that was previously reported by Zhao *et al*., 2001 (Figure 5.2).

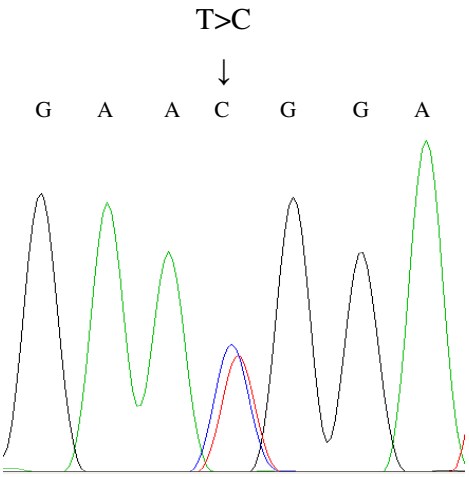


Figure 5.2. Chromatogram displaying the c.941A>G alteration in exon eight of the Atlastin gene in patient H2 in the antisense strand.

Altered migration profiles were detected by SSCP analysis in the second exon of the Atlastin gene in 28 out of 42 patients analyzed (Figure 5.3).

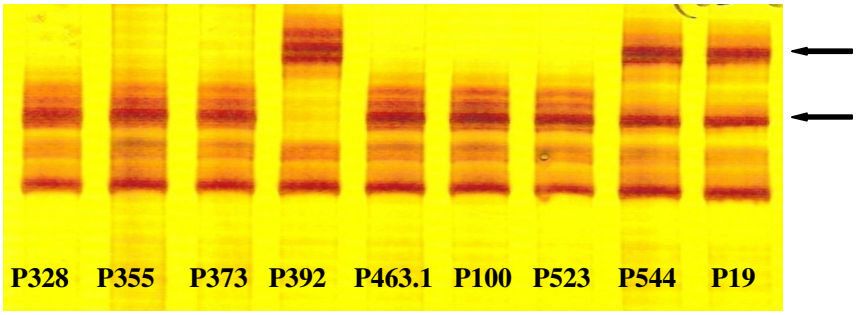


Figure 5.3. Eight per cent SSCP gel with glycerol showing SSC polymorphisms in the second exon of *Atlastin* in patients P392, P544, and P19.

Sequencing analysis of DNA samples from patients P19 and P392 revealed a 84A→G variation (Figure 5.4 and 5.5) that does not change the amino acid proline at position 28. This alteration was previously reported in NCBI as a polymorphism by the reference number 35014209.

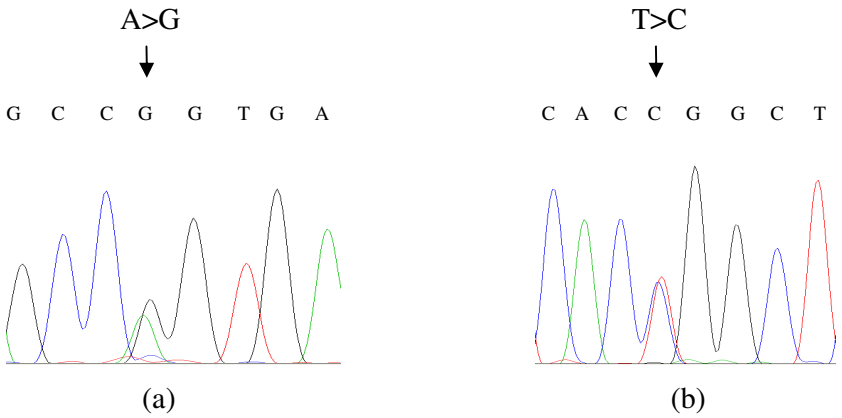


Figure 5.4. Chromatograms showing the heterozygous c.84A>G variation in patient P19 in the second exon of *Atlastin* in the sense (a) and antisense (b) strands.

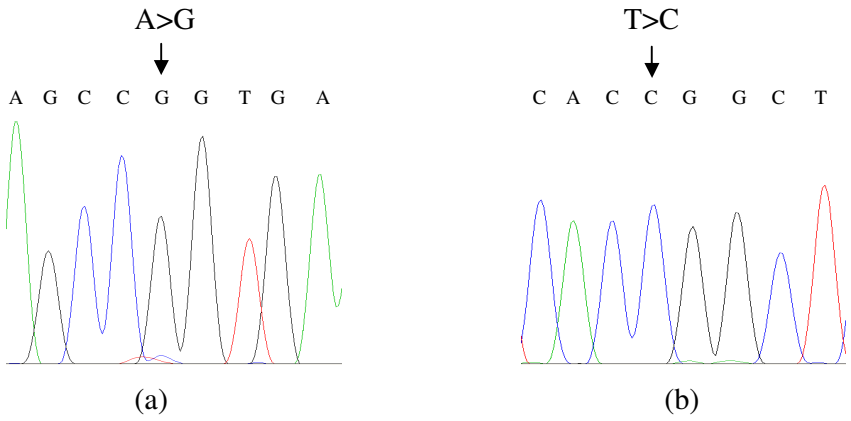


Figure 5.5. Chromatograms displaying the homozygous c.84A>G variation in patient P392 in the second exon of *Atlastin* in the sense (a) and antisense (b) strands.

A different migration pattern was also observed in patient P461.1 in exon three of the Atlastin gene by SSCP analysis (Figure 5.6).

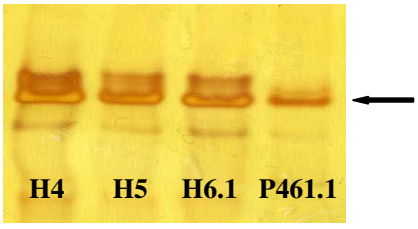


Figure 5.6. Eight per cent SSCP gel with glycerol showing a different banding pattern of exon three of *Atlastin* in patient P461.1.

The following sequence analysis showed a homozygous c.351G>A (p.E117E) alteration in patient P461.1 (Figure 5.7) which was previously reported as a polymorphism in the NCBI by the reference number 1060197.

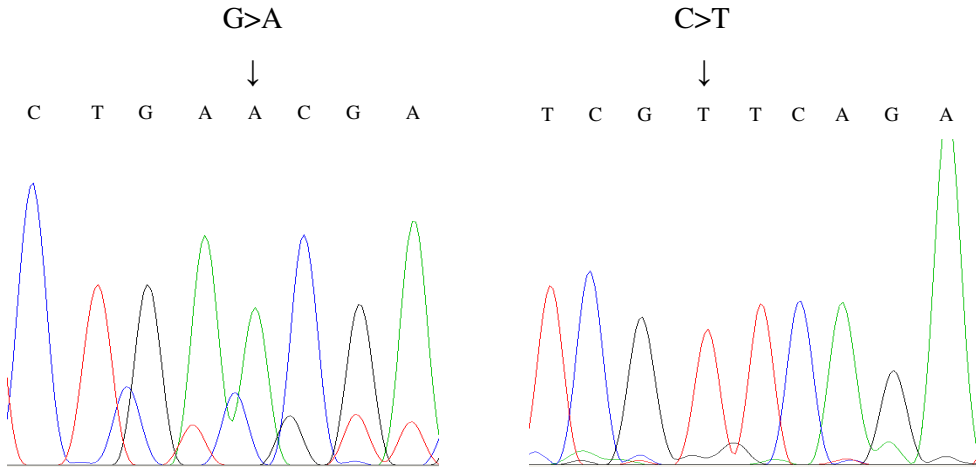


Figure 5.7. DNA sequencing profiles of patient P461.1 displaying the c.351G>A alteration in exon three of the Atlastin gene in the sense (a) and (b) antisense strand.

SSCP analysis of exon six of the Atlastin gene showed an abnormal banding profile for 37 out of 42 analyzed patients (Figure 5.8).

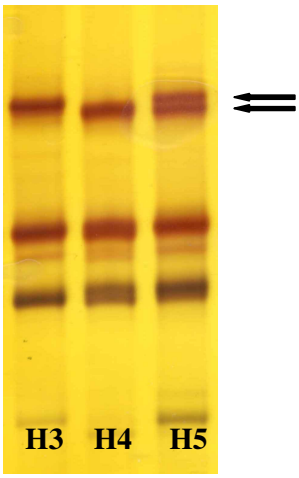
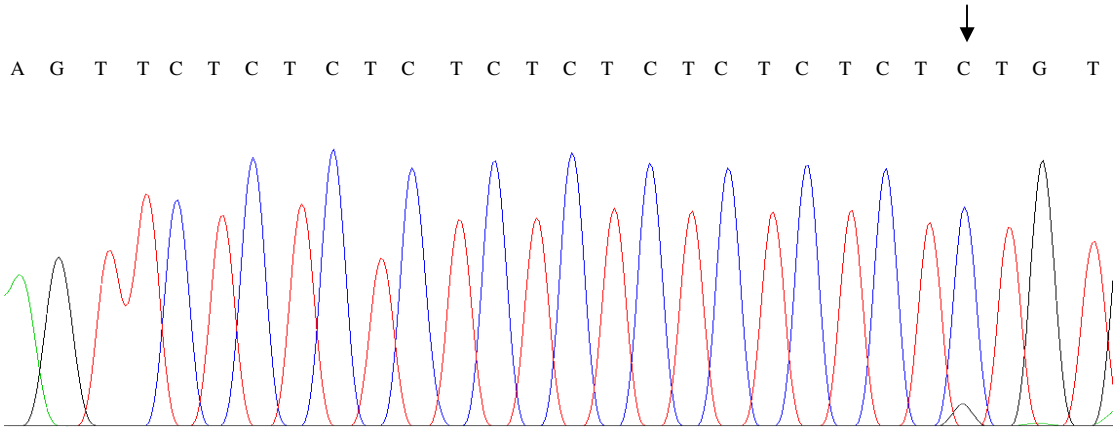
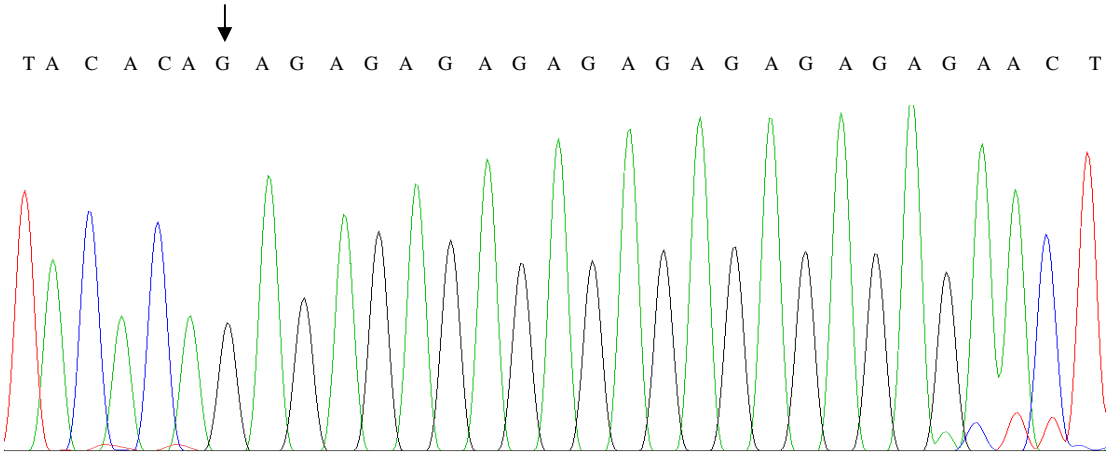


Figure 5.8. Eight per cent SSCP gel with glycerol showing the abnormal banding pattern of exon six of *Atlastin* in patients H4 and H5.

Following sequencing analysis of PCR samples from these patients revealed that patient H4 was homozygous and patient H5 was heterozygous for the g.44569_44570delTC mutation in the non-coding region before exon six of the Atlastin gene. This variation was previously established in NCBI as a polymorphism with a reference number 3834518 (Figure 5.9 and 5.10).

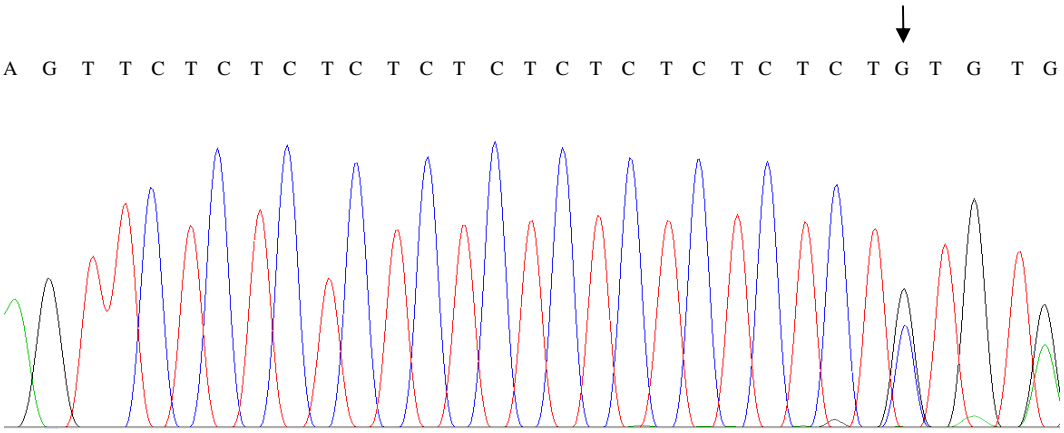


(a)

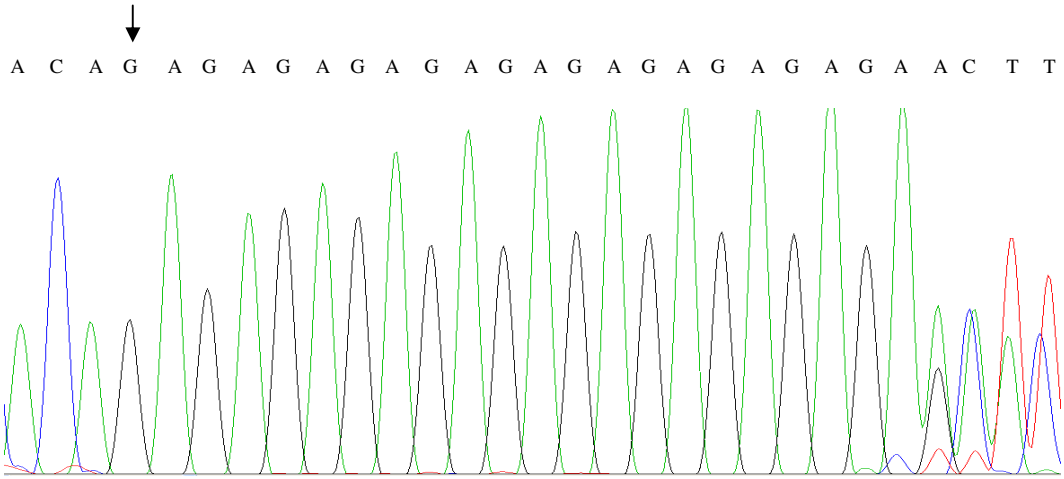


(b)

Figure 5.9. Chromatograms of patient H4 for intronic site five of *Atlastin* showing the g.44569_44570delTC variation in homozygous state in the sense (a) and antisense (b) strands.



(a)



(b)

Figure 5.10. Chromatograms of patient H5 for intronic site five of *Atlastin* showing the g.44569_44570delTC variation in heterozygous state in the sense (a) and antisense (b) strands.

SSCP analysis revealed a different migration pattern in patient H12.1 in exon seven of the Atlastin gene (Figure 5.11).

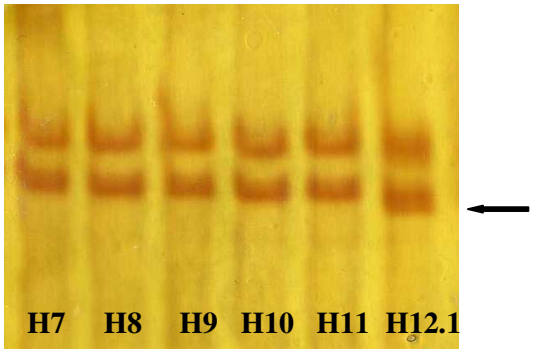


Figure 5.11. Eight per cent SSCP gel indicating the abnormal migration profile of exon seven of the Atlastin gene in patient H12.1.

Sequence analysis displayed a heterozygous c.669C>T substitution in exon seven of the Atlastin gene in patient H12.1 (Figure 5.12). This novel substitution does not alter the tyrosine residue at position 223 and does not create or abolish a restriction site. Thus, in order to screen this novel polymorphism, 28 controls were analyzed by SSCP analysis and none of them were found to carry this alteration.

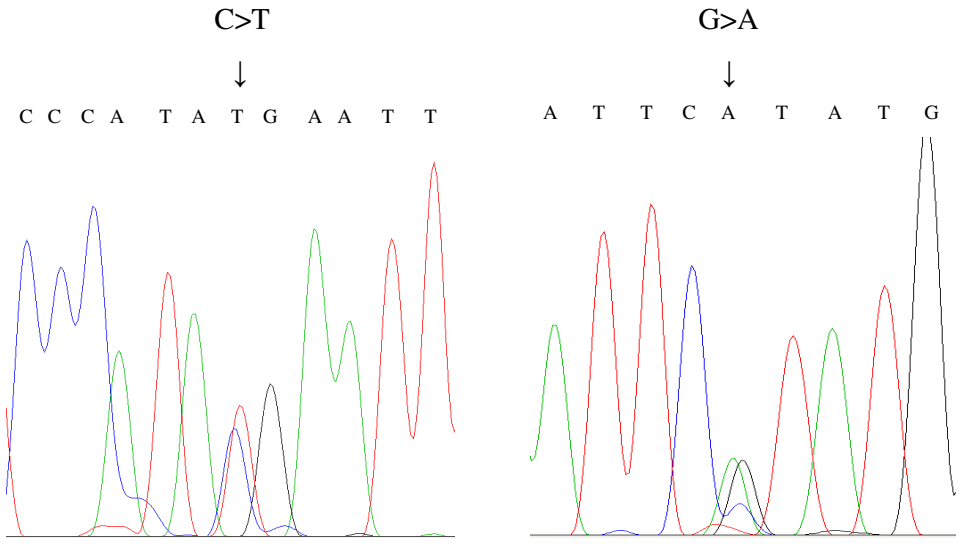


Figure 5.12. DNA sequencing profiles of patient H12.1 for exon seven of *Atlastin* indicating the heterozygous c.669C>T alteration in the sense (a) and antisense (b) strands.

5.1.2. Variations in the Spastin Gene

SSCP analysis of exon six of *Spastin* showed an extra fragment with abnormal migration profile in patient H1.1 (Figure 5.13).

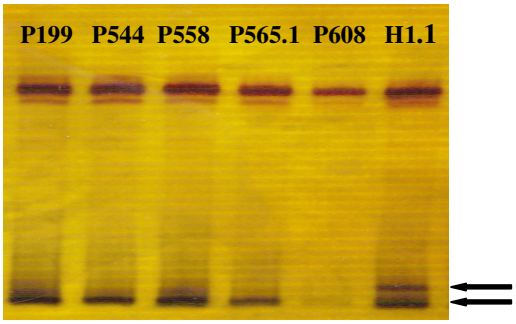
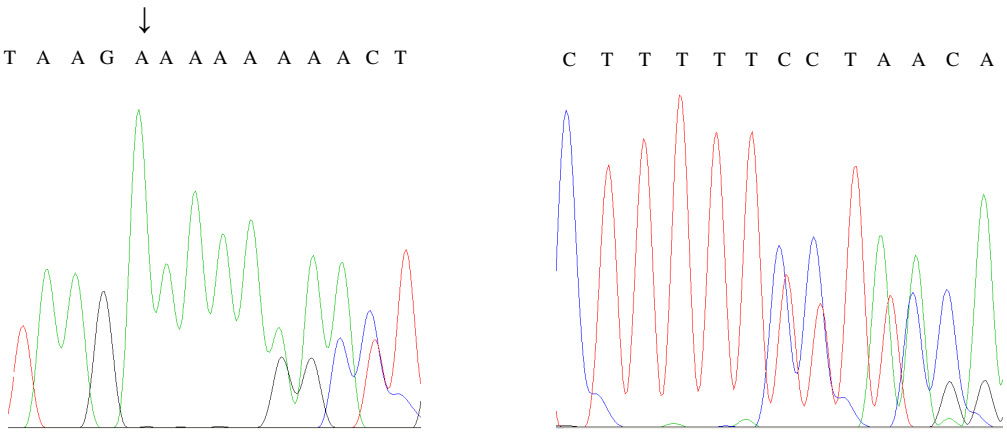


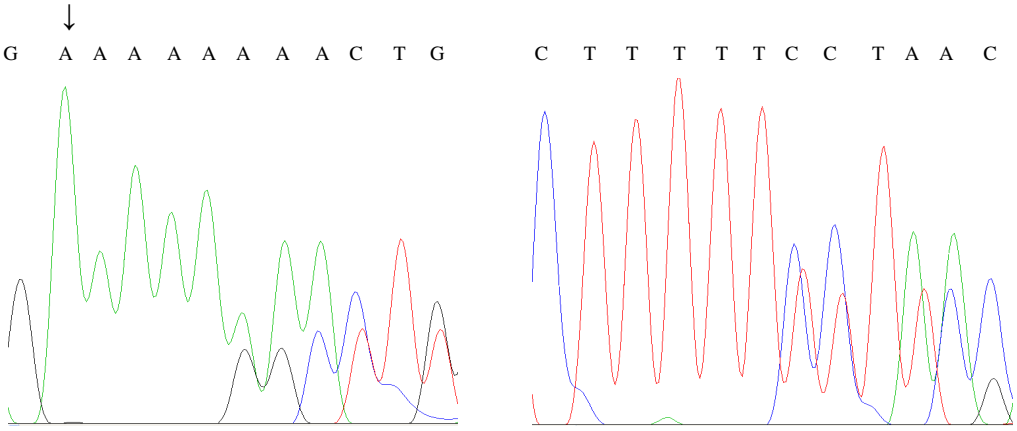
Figure 5.13. Eight per cent SSCP gel with glycerol showing a different banding pattern of exon six of *Spastin* in patient H1.1.

DNA sequencing analysis of patient H1.1 and her father H1.5 revealed a heterozygous one bp insertion at nucleotide position 931 (c.310_311insA) leading to production of a truncated protein (Figure 5.14 and 5.15). This mutation was previously reported by Bantel *et al*., 2001.



(a) (b)

Figure 5.14. Chromatograms showing the c.310_311insA alteration in patient H1.1 in exon six of *Spastin* in the sense (a) and antisense (b) strands.



(a) (b)

Figure 5.15. Chromatograms displaying the c.310_311insA change in patient H1.5 in exon six of *Spastin* in the sense (a) and antisense (b) strands.

SSCP analysis indicated an abnormal migration pattern of exon seventeen of the Spastin gene in two patients, P565.1 and P565.2, from the same family among 42 families analyzed (Figure 5.16).

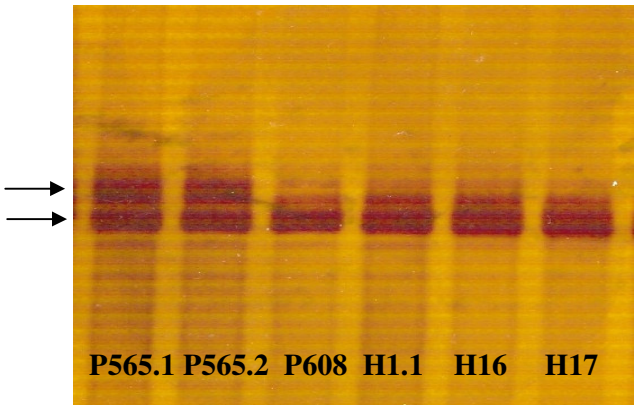


Figure 5.16. Eight per cent SSCP gel with showing a different banding pattern of exon seventeen of *Spastin* in patient P565.1 and his mother P565.2.

Sequencing analysis of patient P565.1 revealed a heterozygous c.1741C>T change (p.Arg581X) leading to a premature stop codon that was previously reported by Patrono et al., 2005 (Figure 5.17). The presence of this alteration was verified by restriction enzyme analysis. The alteration abolishes the restriction site of TaqI, that digests the 241

bp length wild type PCR fragment into 104 bp and 137 bp. TaqI digestion produced 104 bp and 137 bp fragments in controls, whereas the patient's and her son's sample enzyme digestion gave an undigested 241 bp band with 104 bp and 137 bp fragments showing a heterozygous alteration (Figure 5.18).

Figure 5.17. Chromatogram showing the c.1741C>T alteration in heterozygous state in patient P565.1 in the anti-sense strand.

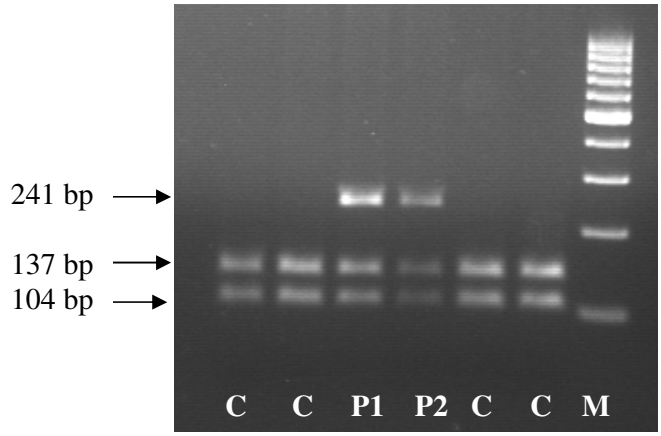


Figure 5.18. TaqI restriction digestion of exon seventeen of the Spastin gene (C: Control; P1: P565.1; P2: P565.2; M: Marker).

A different migration pattern of exon 12 of the Spastin gene was detected in patient P96 by SSCP analysis (Figure 5.19).

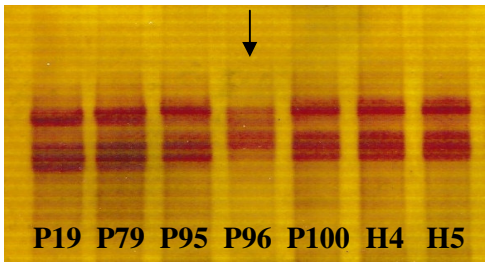


Figure 5.19. Eight per cent SSCP gel with glycerol showing a different banding pattern in exon 12 of *Spastin* in patient P96.

Sequencing analysis of exon 12 for patient P96 revealed a heterozygous IVS12+18G>T substitution in the noncoding region before exon 13 (Figure 5.20). The variation was checked using a splice-site prediction program, Neural Network (Reese *et al*., 1997) (http://www.fruitfly.org/cgi-bin/seq_tools/splice.pl) and it was seen that the alteration does not result in the occurrence of a cryptic splice site. The alteration does not create or abolish a restriction enzyme site. For this reason, in order to understand whether this alteration is a polymorphism or mutation, we performed SSCP analysis with samples of 25 unrelated, healthy people. As a result, we can not determine the alteration in none of the controls.

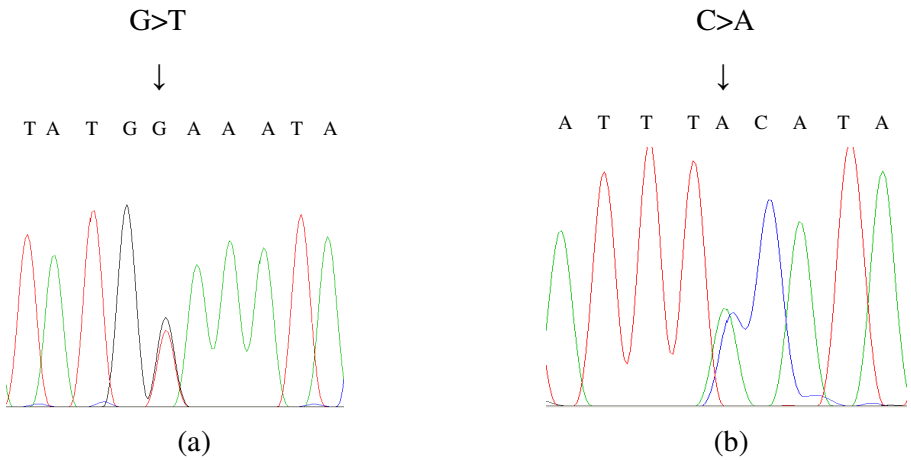


Figure 5.20. Chromatograms displaying the IVS12+18G>T alteration in the non-coding region twelve of *Spastin* in patient P96 in the sense (a) and antisense (b) strands.

5.1.3. Variations in the NIPA1 Gene

A different migration pattern was detected by SSCP analysis in patients P461.1 and her brother P461.2, and P463.1 in exon four of the NIPA1 gene (Figure 5.21).

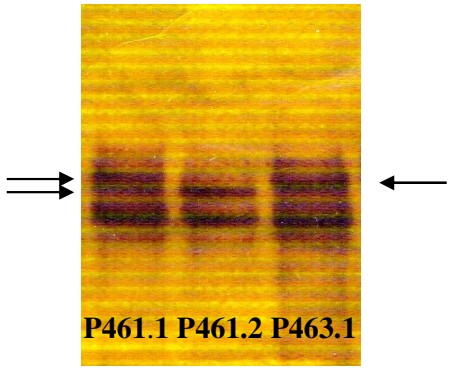
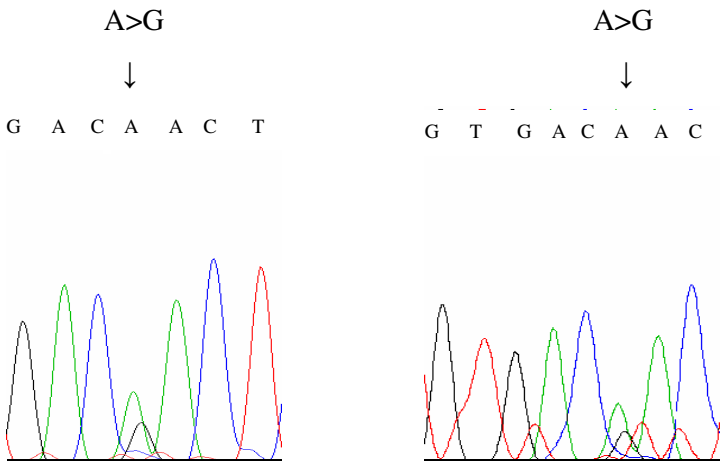


Figure 5.21. Eight per cent SSCP gel showing the abnormal migration profiles of exon four of *NIPA1* in patients P461.1, P461.2, and P463.1.

The following sequencing analysis of these samples revealed a c.444A>G change in exon four of *NIPA1* (Figure 5.22 and 5.23). This alteration does not change the threonine residue at position 147 and was reported as a polymorphism in the database of NCBI with the reference number 11263683.



(a) (b)

Figure 5.22. Chromatograms displaying the heterozygous c.444A>G substitution in patient P461.1 (a) and her brother P461.2 (b) in the sense strand.

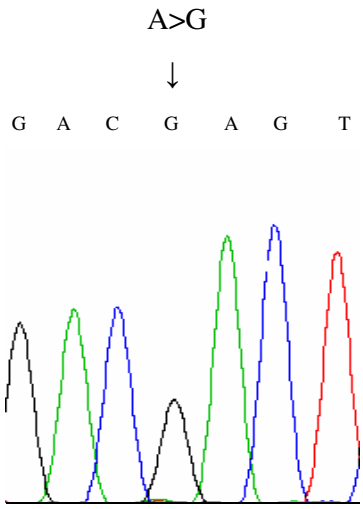


Figure 5.23. Chromatogram indicating the homozygous c.444A>G substitution in patient P463.1 in the sense strand.

The SSCP analysis of exon five of the NIPA1 gene revealed an abnormal banding pattern in 15 out of 42 patients analyzed (Figure 5.24).

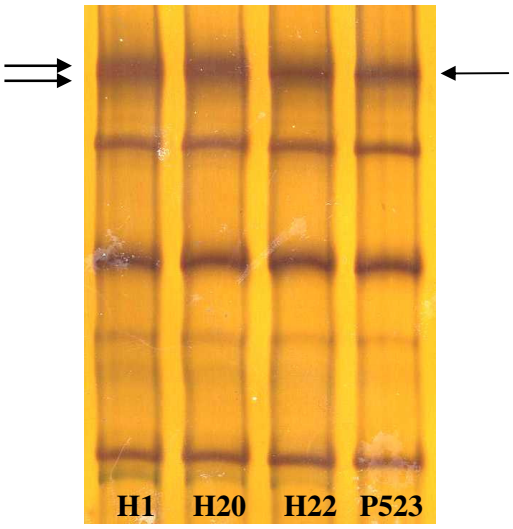


Figure 5.24. Eight per cent SSCP gel with glycerol showing the abnormal banding pattern in exon five of the NIPA1 gene.

The sequence analysis of exon five of *NIPA1* for patient H1.1 revealed a heterozygous IVS4+3236C>T substitution in the noncoding region before exon five of

the gene (Figure 5.25). This variation was previously established in NCBI as a polymorphism with the reference number 12592629.

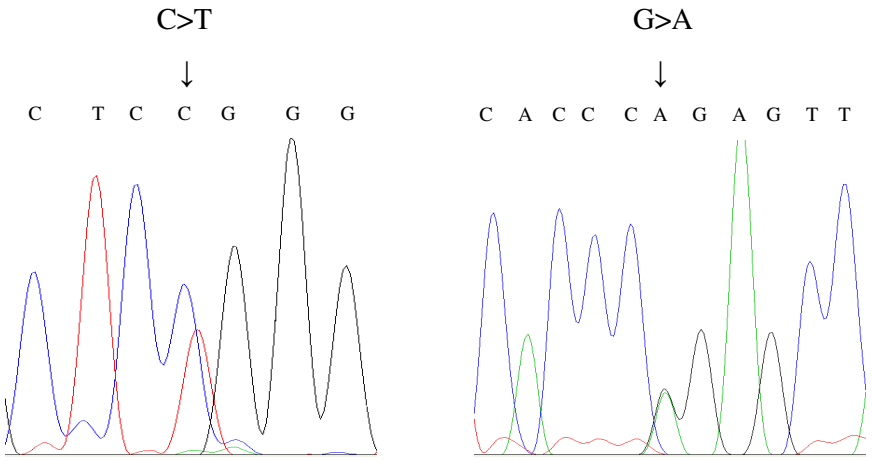


Figure 5.25. Chromatograms showing the heterozygous IVS4+3236C>T alteration in patient H1 in the sense (a) and antisense (b) strands.

5.1.4. Variations in the REEP1 Gene

SSCP analysis revealed a different migration profile of exon four of the REEP1 gene in 30 out of 42 patients analyzed (Figure 5.26).

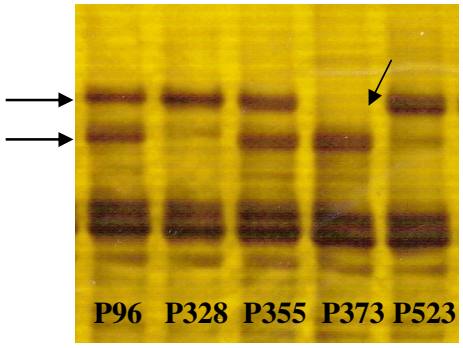


Figure 5.26. Eight per cent SSCP gel showing the different banding pattern of exon four of the REEP1 gene in patients P96 and P373.

Sequencing analysis of exon four of *REEP1* for patients P96 and P373 showed a c.285G>A substitution in the coding region (Figure 5.27) which does not alter the threonine residue at position 95. This variation was previously established in NCBI as a polymorphism with the reference number 2276625.

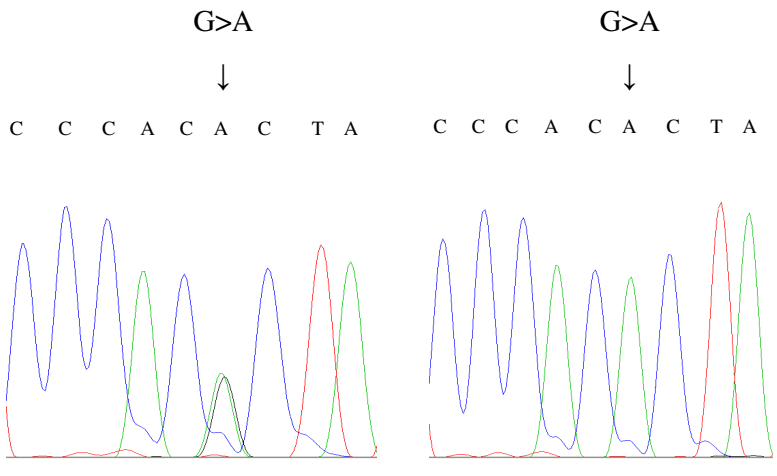


Figure 5.27. DNA sequencing profiles of P96 and P373 indicating that patient P96 was heterozygous (a) and patient P373 (b) was homozygous for the c.285G>A substitution in the exon four of the *REEP1* in the sense strand.

An altered banding pattern of exon five of the REEP1 gene was observed in 11 out of 42 analyzed patients (Figure 5.28).

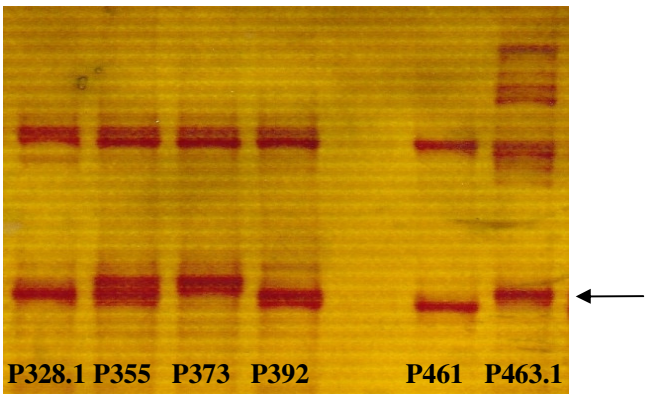
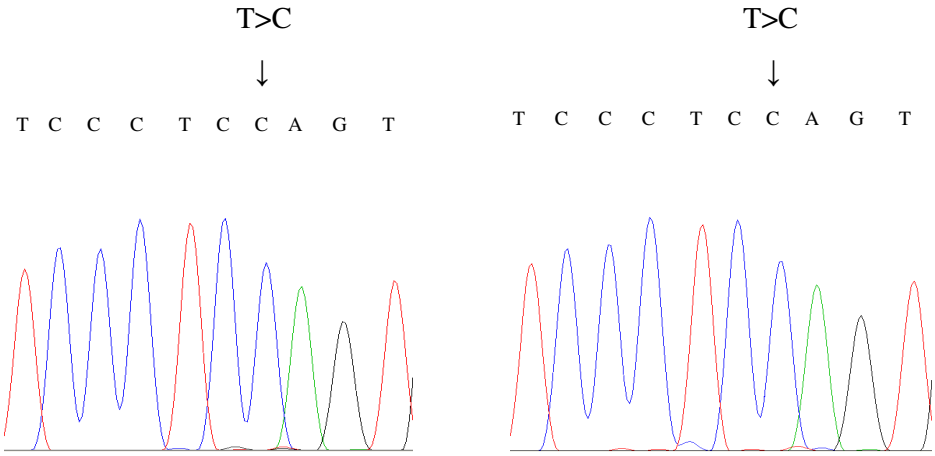


Figure 5.28. Eight per cent SSCP gel displaying the abnormal migration pattern in exon five of *REEP1*.

Sequencing of the PCR samples revealed a homozygous IVS5+45T>C alteration in the noncoding region five in patients P373 and P463.1 (Figure 5.29). This variation was reported in NCBI as a SNP with the reference number 12988844.



(a) (b)

Figure 5.29. Chromatograms indicating the homozygous IVS5+45 T>C alteration in the noncoding region five of *REEP1* in patients P373 (a) and P463.1 (b) in the sense strand.

The sequence alterations identified in this study are shown in table 5.2.

Table 5.2. The sequence variations defined in this study.

Index Patient	Clinical Diagnosis	Gene	Nucleotide Change	Mutation Type	Amino Acid Change
H2	HSP	*Atlastin*	c.941A>G	Missense	p.H258R
P19 P392	CMT I HSP	*Atlastin*	c.84A>G	Polymorphism	p.P28P
P461.1	HSP	*Atlastin*	c.351G >A	Polymorphism	p.E117E
H4 H5	HSP	*Atlastin*	g.44569_44570 delTC	Polymorphism	-
H12.1	HSP	*Atlastin*	c.669C>T	Polymorphism (Novel)	p.Y223Y

Table 5.2. The sequence variations defined in this study (continued).

H1.1	HSP	*Spastin*	c.310_311insA	Frameshift	p.N311fsN
P565.1	HSP	*Spastin*	c.1741C>T	Nonsense	p.Arg581X
P96	CMT V	*Spastin*	IVS12+18G>T	Polymorphism (Novel)	-
P461.1 P463.1	HSP HSP	*NIPA1*	c.444A>G	Polymorphism	p.T147T
H1.1	HSP	*NIPA1*	IVS4+3236C>T	Polymorphism	-
P96 P373	CMT V HSP	*REEP1*	c.285G>A	Polymorphism	p.T95T
P373 P463.1	HSP HSP	*REEP1*	c.412+45T>C	Polymorphism	-

6. DISCUSSION

Hereditary Spastic Paraplegia comprises a clinically and genetically heterogeneous group of inherited disorders. Pure forms of HSP are characterized by spasticity and weakness of the lower extremities, whereas complicated types involve one or more neurological or non-neurological symptoms along with progressive spastic weakness. However, clinical examination may not be enough for the discrimination of subtypes of HSP since symptom onset, disease severity and prognosis can vary between and within families. For this reason, genetic diagnosis is very important for differential diagnosis, genetic counseling, and pre-symptomatic diagnosis of the disease. Furthermore, genotype/phenotype correlations help understanding the disease mechanisms responsible for HSP.

Up to date, 39 loci and 17 genes have been reported to cause HSP. Among them, *Atlastin*, *Spastin*, *NIPA1* and *REEP1* are the most commonly mutated genes and are known to be responsible for more than 50 per cent of autosomal dominant HSP cases. Therefore, we screened these genes in 42 patients as the preliminary step of the genetic analysis of the disease in Turkey. Thirty four of these patients were clinically diagnosed as HSP whereas eight were referred to our laboratory for differential analysis of CMT and HSP phenotypes. As a result of the study, three mutations, one in *Atlastin* and two in *Spastin* were identified.

The *Atlastin* mutation was detected in an HSP patient, patient H2. She was a 46-year-old woman referred to our laboratory with pure HSP diagnosis. She had spasticity in the lower limbs and her reflexes were hyperactive. She was heterozygous for a missense c.941A>G alteration in the Atlastin gene leading to replacement of histidine with arginine at amino acid position 258. This mutation was previously reported by Zhao *et al* (Zhao *et al.*, 2001). They identified this mutation in one family with 16 affected individuals.

Atlastin mutations generally result in SPG3A with early onset. The mutation we found support this notion because the disease started at the age 7-8 in patient H2 who shows pure type HSP with spasticity in the lower limbs. Besides, her pedigree indicates that the disease has an autosomal dominant inheritance since five other family members are affected by the disease. Unfortunately, DNA samples from these family members were not available for genetic analysis.

Atlastin is an integral membrane protein and is a member of dynamin superfamily of guanosine triphosphatases (GTPases). Up to date, more than 20 mutations including commonly missense alterations and one insertion mutation have been identified and most of them result in protein truncation near the C-terminus (Meijer *et al*., 2007). The mutation identified in this study is localized outside of the GTPase domain of atlastin. Among different species although histidine at this position is not conserved, it is always replaced with a basic and polar amino acid. Thus, this alteration may not be expected to impair the activity of the protein. However, the mutation may indirectly impair the GTPase activity of the protein by introducing an abnormal secondary structure. More likely, the mutation may also lead to disease by impairing association of atlastin with other proteins such as spastin (Evans *et al.*, 2006; Sanderson *et al*., 2006) or multimerization.

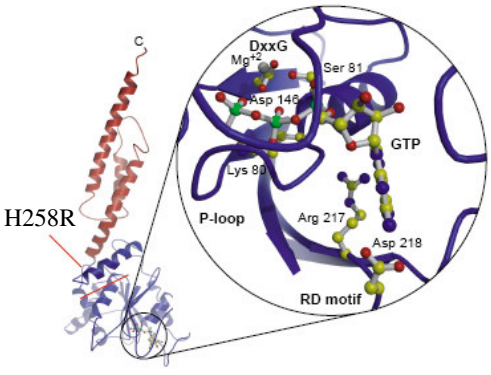


Figure 6.1. Predicted 3D structure of atlastin, GTP bound GTPase active site and the mutation H258R outside of the N-terminal GTPase site (Modified from Zhao *et al*., 2001).

The SSCP analysis of the Atlastin gene also allowed us to detect three previously reported polymorphisms and one novel polymorphism in a total of 42 Turkish patients. First reported polymorphism was a c.84A>G variation in the second exon that does not change the proline at amino acid position 28. According to our SSCP analysis, 27 out of 42 analyzed patients were carrying the polymorphism in a heterozygous state and only one patient, P392, was homozygous for this alteration. The second polymorphism, c.351G>A, was found only in patient 461.1 who was homozygous for the alteration. The polymorphism does not affect the glutamic acid residue at amino acid position 117. The third polymorphism, g.44569_44570delTC, was found in 37 out of 42 analyzed patients.

The novel polymorphism was detected in a 17-year-old male patient, H12.1. He was referred to our laboratory with complicated type of HSP. Sequence analysis displayed a heterozygous c.669C>T substitution in exon seven of the Atlastin gene that does not alter the tyrosine residue at amino acid position 223. This residue is conserved through the species of *Homo sapiens*, *Pan tryglodytes*, *Mus musculus* and *Rattus norvegicus*. The variation does not create or abolish a restriction site. Thus, in order to screen this novel polymorphism, 28 healthy controls were analyzed by SSCP analysis and none of them were found to carry this alteration. It should be screened for in more chromosomes to unravel whether it is causative or have a modifier effect on gene expression.

SSCP analysis of the Spastin gene in 42 patients led to detection of two different mutations in patients H1.1 and P565.1. The first patient, H1.1, was a 18-year-old male. The disease started at the first decade of life. He had abnormal walking, pes cavus and scoliosis. He had no problems with upper limbs indicating that all clinical features were consistent with the pure type HSP. He was heterozygous for a one bp insertion at nucleotide position 931 (c.310_311insA) in exon six of the Spastin gene. According to his pedigree, his parents were not consanguineous and his father and aunt were also affected showing that the disease was inherited autosomal dominantly. Further analysis indicated that his father, H1.5, was carrying the same mutation. However, we had no clinical data about his father.

De Bantel *et al.* previously identified this mutation in a 47-year old male patient with pure HSP (De Bantel *et al.*, 2001), however, it was reported to be at nucleotide position 1062 with the same codon number. Their patient had spasticity in the lower extremities that started to develop at the age of 40. His sister had spastic paraparesis that began in her fourth decade. Furthermore, his half-brother had spastic paraparesis since infancy and was restricted to a wheelchair (De Bantel *et al.*, 2001). Interestingly, the disease started in the first decade of life in our patient H2, but at variable ages in the previously reported family. These two studies confirm that spastin mutations lead to pure SPG4 in which the age of onset and the severity of the disease may be variable suggesting that modifiers have strong effects on the clinical phenotype.

The second patient, P565.1, was referred to our laboratory with pure HSP diagnosis. SSCP analysis of exon seventeen of the Spastin gene in patient P565.1 revealed a heterozygous c.1741C>T alteration (p.Arg581X) leading to a premature stop codon that was previously identified (Patrono *et al.*, 2005). P565.1 was a 46-year-old man who had a prominent spasticity in the lower limbs. He had difficulty in walking since his 20's and it became more apparent after the age of 40. His mother was also affected. She had lower limb spasticity and walking difficulty since her 50's. We also analyzed the mother's DNA sample and identified the same mutation in heterozygous state proving that the disease inherited as an autosomal dominant trait. This second case was another example for age of onset variation even in the same family. On the other hand, anticipation was suspected to be a disease mechanism for SPG4 but contradicting results have been reported (Raskind *et al.*, 1997; Reddy *et al.*, 2007).

The differences between age of onset, disease progression and presence of symptoms are suggested to be the result of the existence of genetic modifiers that modulate the disease phenotype. Besides, the imbalance of the wild type and abnormal proteins in protein complexes required for several cellular processes may be the other reason of these varieties (Depienne *et al.*, 2007).

Spastin protein is composed of 616 amino acids. It is a member of the AAA protein family and is suggested to behave as a chaperone during assembly, disassembly and function of the protein complexes. Up to date, more than 150 mutations were found in

Spastin and disease causing mutations were found to be scattered throughout the gene, except exon four. Most of them cause the disease either by inactivating the functional domain of the protein or reducing the amount of spastin (Fonknechten *et al.*, 2000). Consistently, in patient H1.1, the insertion mutation we found resulted in the formation of truncated spastin proteins. Besides, the c.1741C>T mutation that was found in patient P565.1 affects the AAA domain by altering the amino acid at position 581 and resulting in a premature stop codon that leads to formation of an abnormal protein. The AAA cassette of *Spastin* is confined between amino acids 342 and 599. Thus, these alterations may directly cause to loss of protein activity by inactivating functional AAA domain which in turn leads to decrease the amount of functional spastin. This hypothesis indicates that axonal preservation in the corticospinal tract requires the threshold level of *Spastin* expression. Furthermore, mutant products may show dominant negative effect on normal proteins by interfering with the translation and biological function of the normal protein.

Spastin gene analysis also let us to identify a new polymorphism in patient P96 who was referred to our lab with a diagnosis of CMT with pyramidal signs. This heterozygous IVS12+18G>T substitution was in the noncoding region before exon 13. We checked the variation whether it was a splice site alteration using a splice-site prediction program, Neural Network (Reese *et al*., 1997) revealing that the alteration does not create a cryptic splice site. None of the 25 controls were positive for the variation suggesting that it is not a common polymorphism. Furthermore, his affected brothers, P95 and P100, do not have this alteration suggesting that there is no association between the disease and the nucleotide variation.

SSCP analysis allowed the identification of two known polymorphisms in the Non-imprinted in Prader-Willi/Angelman Syndrome 1 (NIPA1) gene. First polymorphism was a c.444A>G change in exon four of *NIPA1* that does not alter the threonine residue at position 147. The polymorphism was detected in patient P461.1, her brother P461.2 and in another patient, P463.1. The second polymorphism, IVS4+3236C>T, was found in exon five of *NIPA1* in patient H1.1. It was a heterozygous substitution in the non-coding region before exon five of the gene. According to our SSCP analysis, 15 out of 42 analyzed patients were carrying this polymorphism.

Receptor Expression Enhancing Protein 1 (REEP1) gene screening in 42 patients allowed us to detect two reported polymorphisms suggesting that this gene is not commonly mutated in Turkish HSP patients. The first variation was detected in patients P96 and P373. Sequencing analysis of exon four of *REEP1* revealed a c.285G>A substitution in the coding region that does not alter the threonine residue at position 95. Based on our SSCP analysis, 30 out of 42 patients analyzed had this variation. Second known polymorphism, IVS5+45T>C, was found in the non-coding region five in patients P373 and P463.1 that were homozygous for the polymorphism. Eleven of the 42 patients were carrying this polymorphism.

According to the literature, mutations of these four genes are responsible for 50 per cent of pure AD-HSP cases. We have identified only three mutations among 42 unrelated patients, however, subsequent verification of clinical descriptions revealed that 13 patients in our cohort had autosomal dominant pure type HSP. Thus, the frequency of mutations among these 13 patients was found to be 23.7 per cent lower than reported previously. Although *KIAA0196*, *KIF5A, HSP60*, and *ZFYVE27* are not commonly mutated in HSP, they should be also screened in our cohort of patients.

The pedigree analysis revealed that 15 of our cases could have sporadic mutations and 12 have recessive inheritance. For the recessive cases this observation was confirmed by genetic analysis that showed absence of mutations in the four AD/ pure HSP genes. The recessive cases will be further analyzed for specific loci. For the sporadic cases all loci should be analyzed.

Recent studies indicated large exon deletions in the Spastin and REEP1 genes as causative mutations. They are reported to be responsible for 20 per cent of cases that were negative for point mutations (Beetz *et al*., 2006; Depiene *et al*., 2007). These gross mutations can not be identified by standard techniques such as SSCP, DHPLC (denaturing high performance liquid chromatography) or direct sequencing. Recently, in order to detect large rearrangements including one or more exons, multiplex ligation-dependent probe amplification assay was employed and large rearrangements were found to be frequent specifically in SPG4. In our further studies the large exon rearrangements

should also be screened and help to identify the causative mutations in a higher number of patients. DHPLC or direct sequencing can be preferred in our further studies to unravel small mutations rather than SSCP analysis. We performed SSCP analysis because of its advantages in cost, labor, and time. However, SSCP analysis generally detects approximately 80 per cent of nucleotide variations. In the study, we only examined coding exons and flanking sequences of the genes. Alternatively, other regions such as promoter or polyadenylation sites can be studied.

This study is important for understanding the genetic basis of HSP in Turkish population. Our results indicate that Atlastin and Spastin should be the screened initially for genetic analysis, however, NIPA1 and REEP1 mutations might not be common among our AD pure HSP cases. Pedigree analysis suggested that autosomal recessive cases of HSP might be more common in our population. High number of consanguineous marriages in Turkey may support this hypothesis. The study also emphasizes the importance of genetic analysis for the discrimination of subtypes of HSP. Moreover, the identified mutations in these genes will be useful for the unraveling the roles of these genes in the disease pathogenesis.

7. CONCLUSION

This study is important for understanding the genetic basis of HSP disease in Turkish population. Screening of commonly mutated genes; *Atlastin*, *Spastin*, *NIPA1* and *REEP1* in pure autosomal dominant HSP (AD-HSP) might be a preliminary step for genetic dignosis of the disease. Three known mutations and two novel polymorphisms in the Atlastin and Spastin genes were defined in the study. The mutations were suggested to result in the disease by forming truncated proteins. However, the exact mechanism was not defined and in order to decipher their roles in the disease, these mutations should be investigated by functional studies. Moreover, novel polymorphisms should also be analyzed functionally whether they have an effect on age of disease onset or severity of the disease. Identification of low mutation frequency for these genes in our cohort of patients unraveled the necessity of screening other genes known to be responsible for AD-HSP. Furthermore, autosomal recessive forms of HSP might be more common in Turkey and these genes should also be analyzed.

In the study, SSCP technique was employed that is not effective for detection of large exon reaarangements. Since these rearrangements are now known to be responsible for 20 per cent of cases that are negative for point mutations other more sensitive and accurate techniques such as MLPA or quantitative real time PCR should be used in our furher studies.

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