EXCLUSION OF AUTOSOMAL RECESSIVE HEREDITARY SPASTIC PARAPLEGIA LOCI IN TURKISH FAMILIES

by

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For my parents,

I have been the luckiest person with your endless love,

inexhaustible patience and great sacrifice.

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ABSTRACT

EXCLUSION OF AUTOSOMAL RECESSIVE HEREDITARY SPASTIC PARAPLEGIA LOCI IN TURKISH FAMILIES

Hereditary spastic paraplegias (HSPs), also known as Strümpell-Lorrain disease, describe a heterogeneous group of genetic neurodegenerative disorders. The main feature of the disease is progressive spasticity in the lower limbs. HSPs are classified according to the mode of inheritance, which can be autosomal dominant, recessive, or X-linked.

In this study, 15 families with possible autosomal recessive inheritance were analyzed and linkage to AR-HSP loci -SPG5, SPG7, SPG11, SPG15, SPG20 and SPG21- was tested. The genes responsible for the disease have already been identified in these loci. SNP markers that are located on restriction sites were used for haplotype analysis. For one family, linkage to SPG11 and for another family, linkage to SPG15 was observed. In four of the families, linkage to all tested loci was excluded. Among the other ten families analyzed, in four families SNP markers on SPG5, SPG11, or SPG21 were non-informative since both affected and unaffected individuals were homozygous for one of these loci. SPG7 locus markers gave inconclusive results in five families, just as markers in SPG20 locus giving inconclusive results for one family. In two of the families, restriction analyses gave both non-informative and inconclusive results. Patients in two of the families analyzed in this study were found to be homozygous for markers in SPG11 locus. Other loci were excluded in these ten families.

Higher incidence of autosomal recessive types of HSP in the Turkish population with respect to western countries provides a gateway for the identification of causative genes. Since known genes are excluded in the majority of our families, the causative mutations are localized either to unknown loci or to other loci for which the responsible gene has not been identified, yet. Therefore, this study forms a base for future studies that will possibly lead to identification of novel genes.

ÖZET

TÜRK AİLELERİNDE OTOZOMAL ÇEKİNİK HEREDİTER SPASTİK PARAPLEJİ LOKUSLARININ DIŞLANMASI

Herediter spastik parapleji (HSP), Strümpell-Lorrain hastalığı olarak da adlandırılan, kalıtsal nörodejeneratif bir hastalıktır. Hastalığın en belirgin özelliği alt ekstremitelerde giderek artan spastik zaaf ve yürüme güçlüğüdür. Kalıtım türüne göre otozomal baskın, otozomal çekinik ya da X'e bağlı HSP olarak sınıflandırılır. Bugüne kadar 39 lokus ve 16 gen bildirilmiştir.

Bu çalışmada, olası otozomal çekinik kalıtıma sahip 15 aile incelendi ve sorumlu genlerin tanımlanmış olduğu otozomal çekinik HSP lokuslarına -SPG5, SPG7, SPG11, SPG15, SPG20 ve SPG21- bağlantı test edildi. Haplotip analizleri, restriksiyon alanlarında bulunan tek baz polimorfizm (SNP) markörleri kullanılarak yapıldı. Bir aile için SPG11 bölgesine, diğer bir aile için SPG15 bölgesine bağlantı bulundu. Dört ailede, test edilen bütün bölgeler dışlandı. Diğer on aileden dördünde, hasta ve sağlıklı bireylerin SPG5, SPG11 veya SPG21 bölgelerinde incelenen tüm markörler için homozigot bulunması nedeniyle sonuca ulaşılamadı. SPG7 lokusundaki markörler için beş ailede; SPG20 markörleri için ise bir ailede bazlar en az bir markör için tanımlanamadı ve sonuç elde edilemedi. İki ailedeki hasta bireylerin SPG11 lokusundaki markörler için homozigot olduğu belirlendi ama bu ailelerde sağlıklı bireylerden DNA örnekleri bulunmaması nedeniyle bağlantı kesinleştirilemedi. Bu on ailede diğer lokuslara bağlantı dışlandı.

Türkiye'de otozomal çekinik HSP hastalığının batı toplumlarına oranla daha sık görülmesi, yeni genlerin tanımlanması çalışmaları için bir temel oluşturmaktadır. Ailelerimizin büyük bir kısmında bilinen genler dışlandığı için bu ailelerde hastalığa neden olan mutasyonların, henüz tanımlanmamış bölgelerde bulunması olasılığı çok yüksektir. Bu çalışma yeni genlerin belirlenmesi için ileride yapılacak çalışmalara temel oluşturması açısından önemlidir.

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A	Adenine
C	Cytosine
G	Guanine
H	Histidine
I	Isoleucine
L	Leucine
M	Methionine
Q	Glutamine
R	Arginine
T	Thymine
V	Valine
W	Tryptophan
X	Stop codon
Y	Tyrosine

LIST OF ACRONYMS / ABBREVIATIONS

aa	Amino acid
AAA	ATPases associated with various cellular activities
AD	Autosomal dominant
ADHSP	Autosomal dominant hereditary spastic paraplegia
AFG3L2	AFG3 ATPase family 3-like 2
APS	Ammonium peroxodisulphate
AR	Autosomal recessive
ARHSP	Autosomal recessive hereditary spastic paraplegia
ARHSP-TCC	Autosomal recessive HSP with thin corpus callosum
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BMP	Bonw morphogenic protein
bp	Base pair
CAM	Cell adhesion molecule
cM	Centimorgan
CNS	Central Nervous System
CYP7B1	Cytochrome P450, family 7, subfamily B, polypeptide 1
Del	Deletion
DHEA	Dehydroepiandrosterone
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribo Nucleotide Tri-Phosphate
EDTA	Ethylene diamine tetraacetate
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
EtBr	Ethidium bromide
GABA	Gamma-aminobutyric acid
GTPase	Guanidin triphosphatase
HSP	Hereditary Spastic Paraplegia
KIF5A	Kinesin family member 5A

L1CAM	L1 cell adhesion molecule
Mb	Megabase
$MgCl_2$	Magnesium chloride
min	Minute
mRNA	Messenger ribonucleic acid
MIT	Microtubule interacting and endosomal trafficking
MT	Microtubule
NIPA1	Non imprinted in Prader-Wili/Angelman syndrome 1
OD_{260}	Optical density at 260 nm
PCR	Polymerase chain reaction
PLP	Proteolipid protein
PMP22	Peripheral myelin protein 22
PNS	Peripheral nervous system
rcf	Relative centrifugal force
REEP1	Receptor enhancing-expression protein 1
ROS	Reactive oxygen species
Rpm	Revolution per minute
SDS	Sodium dodecyl sulphate
SNP	Single Nucleotide Polymorphism
SPAST	Spastin gene
SPG	Spastic paraplegia gene
SPOAN	Spastic paraplegia, optic atrophy, and neuropathy
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TEMED	N, N, N, N'-Tetramethylethylenediamine
TM	Transmembrane
TMD	Transmembrane domain
TRS	Troyer syndrome
W	Watt
ZFYVE26	Zinc-finger protein with a FYVE domain 26

1. INTRODUCTION

Hereditary Spastic Paraplegia (HSP), also called Strümpell- Lorrain syndrome or Familial Spastic Paraparesis, describe a clinically and genetically heterogeneous group of inherited neurodegenerative diseases (Casari and Rugarli, 2001). First clear description of the disease was made in 1880 by a German neurologist, Adolph Strümpell. French physician Maurice Lorrain described HSP more extensively in 1888 (McDermot *et al*., 2000). Although the prevalence varies in different studies, it is accepted as 3-10 cases per 100 000 population (Salinas *et al*., 2008).

The main clinical features of HSP are progressive weakness and spasticity of the lower limbs, lower extremity hyperreflexia and extensor plantar responses (Fink, 2003). Pathologically, the disease is characterized by retrograde degeneration of the upper motor neurons with longest axons in the corticospinal tract and posterior columns (Crosby and Proukakis, 2002; Salinas *et al*., 2008). Upper motor neurons control lower extremity function and constitute the longest neurons with axons greater than one meter. The mechanism underlying the disease in these cells is called 'dying back' because degeneration starts at the distal ends of the fibers and goes towards the cell body (DeLuca *et al*., 2004). Proteins shown to be involved in HSP, have functions in several important cellular processes such as membrane trafficking, axonal transport, energy utilization, signaling, cytoskeletal organization, and synaptic transmission which are the major processes that the neurons rely on (Salinas *et al*., 2008; Stevanin *et al.,* 2008). On the other hand, though lower motor neurons can also have long axons, they are usually protected in HSP cases (Soderblom and Blackstone, 2006).

HSP is classified as 'pure (uncomplicated)' or 'complicated' according to the presence or absence of additional neurological symptoms. In pure HSPs, generally spastic paraplegia exists exclusively but in some cases, urinary symptoms and pes cavus are observed. Neurological findings included in complicated HSP can be thin corpus callosum, mental retardation or cognitive decline, dementia, deafness, cerebellar ataxia, retinopathy, epilepsy, dysarthria, ichthyosis, optic atrophy, peripheral neuropathy, retinitis pigmentosa, and cataract (Depienne *et al.,* 2007b; Battini *et al.,* 2011; Reid, 1999).

Age of symptom onset and rate of progression are highly variable not only among different types of HSP but also in interfamilial cases (Fink, 2003), showing genetic heterogeneity of the disease. Age of onset can be at infancy or reach up to eight decade (Roşulescu *et al.,* 2009).

HSP is also classified based on the mode of inheritance; as autosomal dominant (ADHSP), autosomal recessive (ARHSP), or X-linked. Autosomal dominant HSP is the most common form with 70-80% frequency in Western countries and are generally associated with pure forms (Depienne *et al.,* 2007b). Conversely, autosomal recessive cases generally have a complex phenotype and are more frequent in Mediterranean and North African countries and inbred populations (Blackstone *et al.,* 2011). X-linked HSP is a rare subtype; however, compared to other types its genetic basis is well understood (McDermot *et al*., 2000). To date, at least 39 spastic paraplegia gene (*SPG*) loci have been mapped and 16 genes have been identified (Salinas *et al*., 2008).

1.1. Autosomal Dominant HSP

Eighteen loci have been mapped and eight genes have been identified responsible for autosomal dominant HSP (ADHSP). SPG3A and SPG4 are the most common types of ADHSP, responsible for 50% of the mutations identified. The molecular genetics and the impact of these loci have been described in the following sections.

1.1.1. SPG3A

The first identified autosomal gene that causes HSP is SPG3A which is located on chromosome 14q11-q21 and contains 14 exons (Dürr *et al.,* 2004). The gene encodes for a 558–amino acid protein, Atlastin, that belongs to the dynamin family of large guanidin triphosphatases (GTPases).

Atlastin mutations are the second most common cause of ADHSP, with approximately 10% frequency (Fink *et al.,* 1996). These mutations are associated with an early-onset, pure HSP phenotype and responsible for approximately 30%-50% of such cases (Abel *et al.,* 2004; Dürr *et al.,* 2004).

Atlastin, also called as Atlastin-1, is an integral multimeric membrane protein that is composed of an N-terminal GTPase domain containing three conserved GTP-binding motifs (P-loop, DxxG and RD) and two C-terminal transmembrane (TM) domains (Dürr *et al.,* 2004; Praefcke and McMahon, 2004). Atlastin subfamily includes two additional atlastin members (atlastin-2, and atlastin-3) in humans (Park *et al.,* 2010). Atlastin-1 is highly expressed in the central nervous system, especially enriched in pyramidal neurons in the cerebral cortex and hippocampus (Smith et al., 2009). On the other hand, expression of atlastin-2 and atlastin-3 are mostly observed in other nonneural tissues (Rismanchi *et al.,* 2008; Zhu *et al.,* 2003). At the subcellular level, strong localization of atlastin in the endoplasmic reticulum and Golgi apparatus is visualized by confocal immunofluorescence microscopy. (Namekawa et al., 2007; Zhu et al., 2006).

To date, more than 30 atlastin mutations have been reported, mainly missense changes that supported a gain of function pathogenic mechanism (Smith et al., 2009; Alvarez *et al.,* 2010). Mutations in the SPG3A gene are mainly clustered around the GTPase domain, causing a decrease in GTPase activity (Botzolakis et al., 2011). Mutant proteins with impaired GTPase function tend to oligomerize with wild-type protein, leading to a dominant-negative effect (Smith et al., 2009). As a result, abnormal ER morphogenesis and impaired vesicle trafficking are observed (Rismanchi *et al.,* 2008; Namekawa et al., 2007).

Homotypic membrane fusion is an essential step for ER biogenesis and maintenance and this process requires GTP hydrolysis. Hu *et al.* (2009) showed that atlastin localizes on ER membranes and functions in this membrane fusion process in a GTP-dependent fashion using yeast homolog of atlastin (Sey1p). Orso *et al.* (2009) provided further evidence for its function in Drosophila using Atlastin fly homologue. It was also shown that atlastin isoforms associate with members of reticulons and DP1 protein families that are required for proper formation of ER tubules (Barlowe, 2009; Voeltz *et al.,* 2006). Therefore, defects in ER morphogenesis are considered as a novel neuropathogenic mechanism that can be affective during development, consistent with the early-onset phenotype of *SPG3A*-associated HSP (Hu *et al.,* 2009).

SPG6 is another adult-onset pure HSP-related gene (Bien-Willner et al., 2006) and its product, NIPA1 may function as an intracellular magnesium transporter (Goytain et al.,

2007). NIPA1 also acts as an inhibitor of bone morphogenic protein (BMP) signaling, which is a critical step for distal axonal function (Tsang et al., 2009). Botzolakis et al. (2011) showed that Atlastin-1 and NIPA1 are direct binding partners and their co-expression is critical for endogenous expression and trafficking of these proteins, by using a combination of immunoprecipitation, confocal microscopy, and flow cytometry methods. They also showed that mutations in atlastin-1 have a dominant negative effect on wild-type NIPA1 and atlastin-1 and NIPA1 may function in the same biochemical pathway.

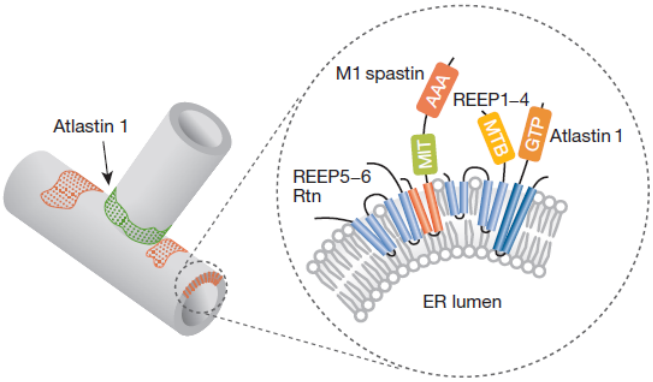


Figure 1.1. Schematic representation of the tubular endoplasmic reticulum, showing ER-related HSP proteins (adapted from Park and Blackstone, 2010).

1.1.2. SPG4

SPG4/SPAST is the most frequent form of HSP, accounting for approximately 40% of the familial and 6-15% of the sporadic cases (Reid, 2003; Alvarez *et al.*, 2010). SPG4 is mostly related with variable-onset, pure HSP although a complicated type has been reported (McDermott et al., 2006).

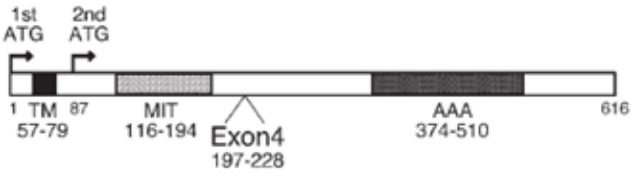
SPG4 gene codes for a 616 amino acid protein, spastin, that belongs to the ATPase associated with various cellular activities (AAA) protein family (Salinas et al., 2007). The gene is located on chromosome 2p22-p21, on a 90 kb region and contains 17 exons (Hazan et al., 1999).

To date, more than 300 spastin mutastions have been identified (Klimpe et al., 2011). They might be missense, nonsense, frameshift, splice site mutations and less frequently, large scale deletions (Depienne et al., 2007a).

Spastin contains two domains: one microtubule (MT)-interacting and endosomal trafficking (MIT) domain at the N-terminus and an AAA domain at the C-terminus (Ciccarelli et al. 2003; Salinas et al., 2005).

Spastin protein is related to p60 katanin and interacts transiently with microtubules (Penny and McCabe, 2005; Errico et al. 2002). Like other AAA ATPases, spastin use the energy of ATP to severe and disassemble microtubules and is required for axon outgrowth and branch formation in neurons (Mecak and McNally, 2010).

Claudiani et al. (2005) showed that SPG4 contains two different translational start sites to synthesize either a full-length isoform (called M1) or a slightly shorter isoform (M85 in rodents or M87 in humans). Besides, Solowska et al. (2008) reported that M1 isoform is more abundant in brain and in spinal cord than in other tissues. They also showed that truncated form of mouse M1 has an adverse effect on axons, while the corresponding truncated form of M85 has no effect on axons. Absence of exon 4 leads to formation of other spastin isoforms (Figure 1.2) (Salinas et al., 2005). Spastin variant lacking exon 4 is found to be present in higher levels in brain and spinal cord (Svenson et al., 2001b). Furthermore, none of the spastin mutations was observed in exon 4 (Salinas et al., 2007). In accordance with these findings, mutations that affect the M1 isoform without exon four are known to be causative in ADHSP.



spastin isoform	MW (kDa)
1st ATG; +Ex4	67.2
2nd ATG; +Ex4	58.0
1st ATG; ΔEx4	63.6
2nd ATG; ΔEx4	54.4

Figure 1.2. Schematic representation of spastin. TM and MIT are shown. Different isoforms of spastin with MWs are shown. (adapted from Salinas et al., 2010).

Haploinsufficiency was proposed as the pathogenic mechanism of SPAST mutations (Alvarez et al., 2010; Solowska et al., 2010). However, leaky splice-site mutations were shown to produce aberrant splice transcripts along with wildtype transcripts, in some HSP

patients. Since the functional spastin may be higher than the expected 50% in these patients a ‘‘thresholdeffect-model’’ was suggested as the pathogenic mechanism rather than haploinsufficiency (Klimpe et al., 2011; Svenson et al., 2001a; Pantakani et al., 2008).

Spastin and atlastin were shown to be binding partners by yeast two-hybrid, GST pull-down and co-immunoprecipitation experiments suggesting that these two proteins might function in a common pathway that is important in axon maintenance (Evans et al., 2006; Sanderson et al., 2006).

The clinical features and the protein products of autosomal dominant HSP loci are summarized in Table 1.1.

Table 1.1. Autosomal dominant forms of HSP (modified from Salinas *et al.*, 2008; Stevanin *et al.*, 2008).

Locus	Chromosome region	Gene (Protein)	Function of the protein	Frequency of mutations	Clinical features
SPG3A	14q12-q21	*SPG3A* (atlastin-1)	GTPase, ER to Golgi transfer, spastin partner	10% of ADHSP	Early-onset pure, slow progression HSP
SPG4	2p22	*SPG4 (spastin)*	Microtubule-severing activity, early secretory pathway	40% of pure AD HSP	Variable-onset mainly pure HSP
SPG6	15q11.1	*NIPA1*	Mg2+ transporter, endosomal trafficking	<1% (9 families)	Adult-onset pure HSP
SPG8	8q24	*KIAA0196 (strumpellin)*	Spectrin domain protein	8% (6 families)	Adult-onset pure HSP, marked spasticity
SPG9	10q23.3-q24.2	*Unknown*		1 family	Cataracts, motor neuropathy, skeletal abnormalities, gastrooesophageal reflux
SPG10	12q13	*KIF5A*	Kinesin heavy chain motor protein	3% (7 families)	Early-onset pure HSP, can be complicated with distal amyotrophy

Table 1.1. Autosomal dominant forms of HSP (modified from Salinas *et al.*, 2008; Stevanin *et al.*, 2008) (continued).

Locus	Chromosome region	Gene (Protein)	Function of the protein	Frequency of mutations	Clinical features
SPG12	19q13	Unknown		<10 families	Early-onset pure HSP
SPG13	2q24-q34	*HSP60*	Mitochondrial chaperone	2 families	Adult-onset pure HSP
SPG17	11q12-q14	*BSCL2* (seipin)	ER integral protein	<20 families	Silver syndrome: variable onset, distal amyotrophy in hands more than in feet
SPG18	Reserved	Unknown			
SPG19	9q33-q34	Unknown		1 family	Adult-onset pure HSP
SPG29	1p31-p21	Unknown		1 family	Sensorineural deafness, hiatus hernia, pes cavus, hyperbilirubinaemia
SPG31	2p12	*REEP1*	Endosomal trafficking, mitochondrial chaperone	8% of AD pure HSP	Variable-onset pure HSP
SPG34	Reserved	Unknown			
SPG36	12q23-q24	Unknown			
SPG37	8p21.1-q13.3	Unknown		1 family	Variable-onset pure HSP
SPG38	4p16-p15	Unknown		1 family	Distal amyotrophy (Silver syndrome)
SPG41	11p14.1-p11.2	Unknown			

1.2. Autosomal Recessive HSP

Autosomal recessive HSP (ARHSP) is generally associated with a complicated phenotype and more common in inbred populations (Blackstone *et al.*, 2011). Eighteen loci

have been mapped and seven genes have been identified responsible for this mode of inheritance in HSP (Table 1.3).

1.2.1. SPG5

SPG5 locus was first mapped to chromosome 8 (Hentati et al., 1994) and subsequently, was narrowed down to an 11-cM interval (Coutinho et al., 1999; Wilkinson et al., 2003; Muglia et al., 2004). By sequence analysis of the genes in this interval, mutations in the CYP7B1 (cytochrome P450, family 7, subfamily B, polypeptide 1) was shown to be associated with HSP (Tsaousidou et al., 2008).

Minority of the ARHSP loci, including SPG5, SPG24, and SPG28, is associated with pure HSP (Muglia et al., 2004; Bouslam et al., 2005). However, in a small number of SPG5-associated HSP cases, additional neurological symptoms indicating a complicated phenotype have been reported (Klebe et al, 2007; Goizet et al., 2009; Arnoldi et al., 2011). These symptoms include mild cerebellar ataxia and optic atrophy (Schüle et al., 2009a; Arnoldi et al., 2011). In addition, white matter abnormalities are observed in several patients with *CYP7B1* mutations (Biancheri et al., 2009; Criscuolo et al., 2009). Age of onset varies from four to 47 years in SPG5-related HSP and patients have a mild to severe phenotype (Goizet et al., 2009). Table 1.2 summarizes the CYP7B1 mutation frequencies.

Table 1.2. Summary of the CYP7B1 mutation frequencies compiled from different studies (modified from Arnoldi et al., 2011).

Total number of cases	Number of families (AR)	Number of sporadics	Number of mutated families (%)	Number of mutated sporadics (%)	Pure familial mutants/ total *n*	Complex familial mutants/ total *n*
403	148	247	16/148 (10.8)	7/247 (2.8)	12/73 (16.4%)	3/63 (4.7%)

Protein product of CYP7B1 is a cytochrome P450 7α-hydroxylase that functions in cholesterol metabolism (Goizet et al., 2009). Although liver mainly make use of another 7α-hydroxylase, CYP7A1, to convert cholesterol to bile acids, CYP7B1 offers an alternative pathway for primary bile acid production (Russell, 2003). Specifically, 7α-hydroxylation of the oxysterol 27-hydroxycholesterol is catalyzed by this enzyme (Russell,

2003). Moreover, CYP7B1 is predominantly expressed in extra-hepatic tissues, including the brain, and provides the primary matabolic route for metabolism of dehydroepiandrosterone (DHEA) neurosteroids and oxysterols (Wu et al., 1999; Rose et al., 1997).

Cholesterol is a critical substance for the proper functioning of the brain and therefore, its levels are tightly regulated. Blood-brain barrier prevents passage of cholesterol between brain and plasma, unless it is converted to side chain oxidized oxysterols (Meaney et al., 2007). The oxysterol 27-hydroxycholesterol, substrate of CYP7B1, was shown to pass from circulation into central nervous system (Heverin et al., 2005). Reduced CYP7B1 mRNA levels in blood (Criscuolo et al., 2009) and increased 27-hydroxycholesterol levels in both plasma (5–9 fold) and cerebrospinal fluid (30- to 50-fold) (Schüle et al., 2009b) were reported in *CYP7B1*-mutated patients. Together, these findings indicate that aberrant oxysterol levels might be the underlying pathogenic mechanism in SPG5-associated HSP (Tsaousidou et al., 2008).

Pettersson et al. (2008) suggested that CYP7B1 has a role in metabolism of 5α-androstane-3β, 17β-diol (3β-Adiol) and 3α-Adiol to triols. Since steroid 3α-Adiol functions in modulating $GABA_A$ receptors, whose ligand GABA is a major inhibitory neurotransmitter in the central nervous system, $GABA_A$-related neuronal pathways are possibly affected from mutant CYP7B1 (Siam et al., 2011).

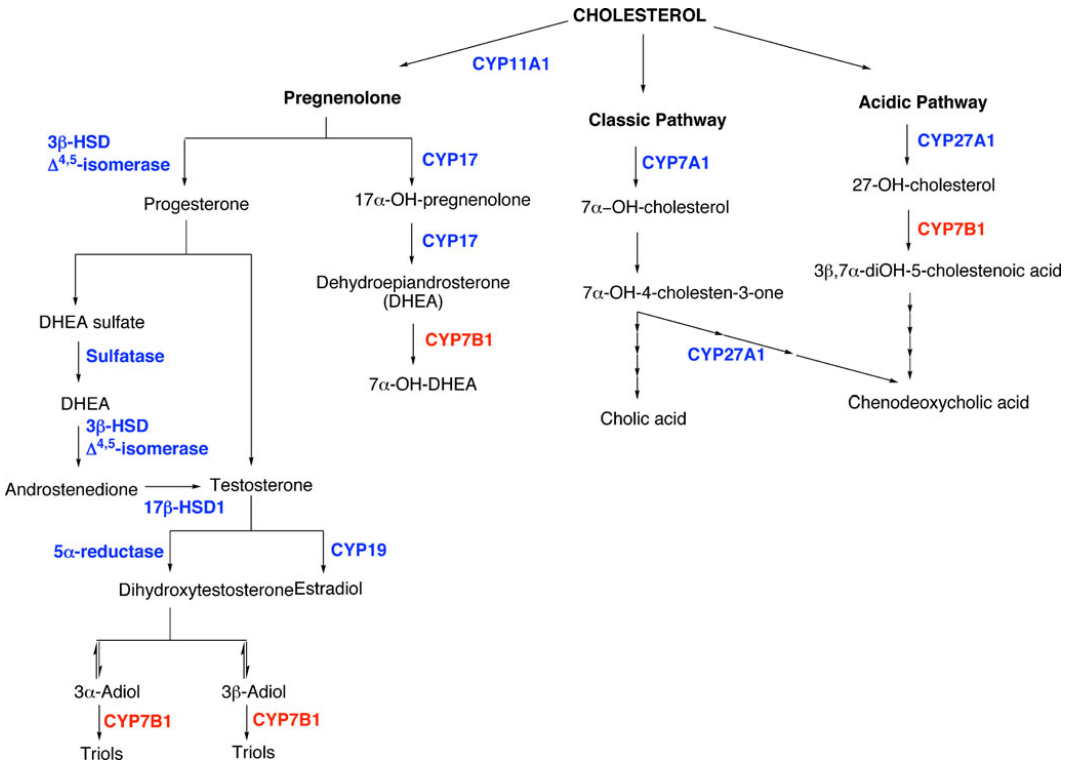


Figure 1.3. Metabolic pathways of cholesterol (adapted from Siam et al., 2010).

1.2.2. SPG7

SPG7 locus has been mapped to chromosome 16q24.3 (De Michele et al., 1998). Age of onset is variable and patients may have pure or complicated phenotype (Wilkinson et al., 2004). SPG7 gene encodes for a 795 amino acid-protein, Paraplegin, which is a mitochondrial zinc metalloprotease belonging to the AAA (ATPase associated with diverse cellular activities) family (Casari et al., 1998). Paraplegin cleaves mitochondrial ribosomal protein MrpL32 that functions in controlling protein quality and regulating ribosomal assembly (Nolden et al., 2005, Koppen et al., 2007). Proper processing of MrpL32 is essential for respiration.

Banfi et al. (1999) identified a paraplegin-related protein, AFG3L2 (ATPase family gene 3-like 2), which localizes to mitochondria, like paraplegin (Casari et al., 1998). Afg3p and Rca1p, yeast homologs of paraplegin and AFG3L2, have a protease activity to degrade mitochondrial translation products by using ATP and a chaperone-like function to help the assembly of membrane-associated ATP synthase (Arlt *et al.*, 1996).

Atorino et al. (2003) showed that paraplegin and AFG3L2 assemble into a high molecular mass complex (900 kD) in the mitochondrial inner membrane. In patients with paraplegin-mutations, an aberrant small complex (250 kD) is observed. Absence of this complex, due to mutations in paraplegin, causes a remarkable reduction in activity of respiratory complex I (NADH–ubiquinone oxidoreductase) in mitochondria (Wilkinson et al., 2004). Reduced complex I activity gives rise to enhanced ROS (reactive oxygen species) production (Li et al., 2003) and an increased sensitivity to oxidative stress (Arnoldi et al., 2008). Increased levels of ROS are suggested to be pathogenic causing paraplegin-associated HSP (Atorino et al., 2003).

Ferreirinha et al. (2004) developed a paraplegin deficient mouse model which is affected by distal axonopathy of spinal and peripheral axons, characterized by axonal degeneration. They reported the presence of abnormal mitochondria in nerve terminals as the first pathological sign and decreased ATP synthesis is observed at later stages of the disease. In addition, alterations in oxidative stress depending on age predominantly affect complex I (Davey et al., 1998). Together with this information, Gelbard (2004) suggested a possible mechanism for the neurologic abnormalities in these animals (Figure 1.4). According to this suggestion, proton gradient between the matrix and intermembrane space is decreased due to abnormal functioning of complex I and as animal ages, the total effect may cause impairment in neurotransmitter release, pathologic generation of reactive oxygen species (ROS), and decreased retrograde transport of trophic substances (Gelbard, 2004).

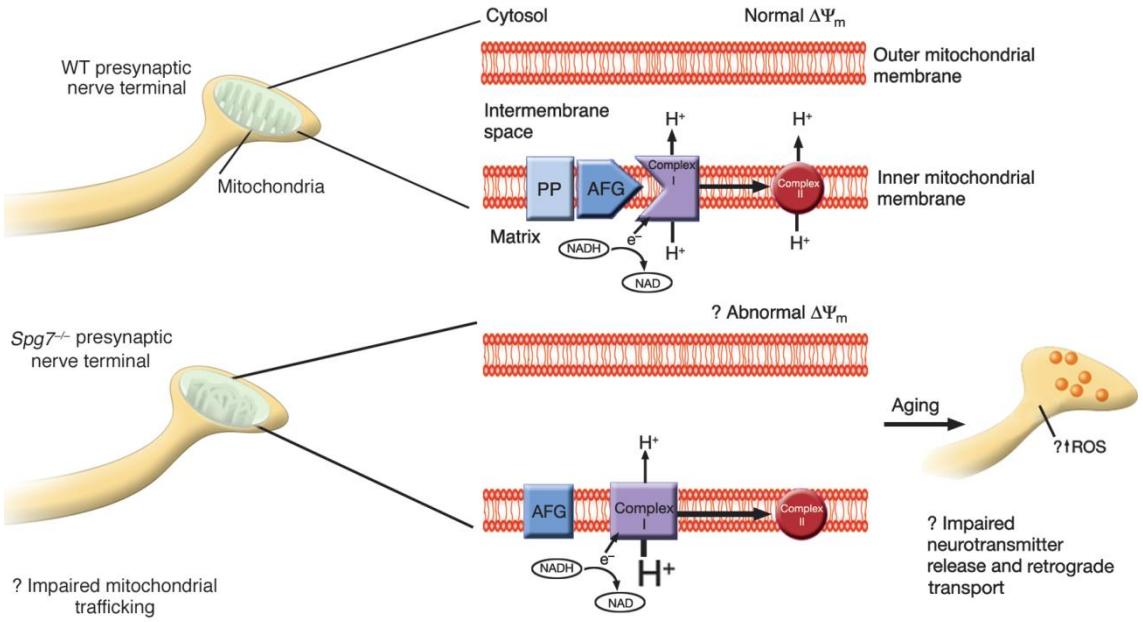


Figure 1.4. Suggested mechanism for decreased complex I activity in presynaptic nerve terminals of *Spg7–/–* mice, (adapted from Siam et al., 2010) (upper part showing the situation with normal paraplegin, lower part indicating the mutant paraplegin case).

Atorino et al. (2003) and Pirozzi et al. (2006) showed that exogenous expression of paraplegin via transfection of cells with a paraplegin-expressing vector halted the progression of neuropathological changes and rescued mitochondrial morphology in the peripheral nerves of paraplegin-deficient mice, suggesting an effective therapeutic option for paraplegin-associated HSP patients.

1.2.3. SPG11

SPG11, located on chromosome 15q13-15 (Winner et al., 2004), is the most common form of HSP with 41% to 77% of all autosomal recessive HSP with a thin corpus callosum (ARHSP-TCC) (Stevanin et al., 2008). SPG11-associated HSP progress slowly and display additional symptoms including urinary incontinence, late distal amyotrophy, cerebellar ataxia and mental deterioration beginning in the second decade of life (Lossos et al., 2006). SPG11 gene, also known as KIAA1840, codes for the protein, spatacsin (Stevanin et al., 2007). This 2,443 amino-acid-long protein show no homology with known proteins but it is highly conserved among vertebrates (Denora et al., 2009).

To date, many different mutations, including nonsense, missense and splice-site mutations, small deletions, small duplications, insertions and large deletions (8,2 and 9 Kb) identified in SPG11 gene (Bauer et al., 2008; Denora et al., 2009). These mutations cause production of truncated protein, suggesting a loss-of-function mechanism (del Bo et al., 2007; Liao et al., 2008).

In two recent studies, SPG11-linked ARHSP-TCC is associated with Juvenile Parkinsonism indicating that SPG11 mutations may cause Parkinsonism in addition to ARHSP-TCC (Anheim et al., 2009; Guidubaldi et al., 2011). Additionally, SPG11 is linked with autosomal recessive juvenile amyotrophic lateral sclerosis, showing that SPG11 mutations cause a wider clinical spectrum than previously observed (Orlacchio et al., 2010).

Although the function of spatacsin remains undetermined, it is predicted to contain a Leucine zipper motif and a Myb domain suggesting a function in regulation of gene expression (Paisan-Ruiz et al., 2008). Spatacsin also includes transmembrane domains showing a possible role in endocytic membrane trafficking (Paisan-Ruiz et al., 2008).

Murmu et al. (2011) showed that spatacsin is expressed in central nervous system, predominantly in cortical and spinal motor neurons, retina and hippocampus, as a possible indicator of dementia, mental retardation and cognitive impairment in HSP patients. They also reported that spatacsin partially co-localizes with various organelles, particularly with protein-trafficking vesicles, endoplasmic reticulum and microtubules. A slight spatacsin expression is detected in corpus callosum, which may explain observation of TCC in SPG11-associated HSP (Murmu et al., 2011). Moreover, the protein is ubiquitously expressed in embryos, indicating a critical role during early neural development (Southgate et al., 2010; Murmu et al., 2011).

1.2.4. SPG15

SPG15 causes the second most common form of ARHSP (Boukhris et al., 2008). SPG15 is located on chromosome 14q22–q24 (Hughes et al., 2001) and is associated with early onset HSP that show severe progression, mental retardation, maculopathy, distal amyotrophy, and mild cerebellar signs (Elleuch et al., 2007). SPG15 codes for spastizin

(ZFYVE26), a highly conserved zinc-finger protein with a FYVE domain (Hanein et al., 2008). FYVE-finger proteins generally interact with various forms of phosphoinositides that function in regulating endocytic membrane trafficking (Gillooly et al., 2001).

Expression profile of spastizin closely resembles that of SPG11. They are both distributed in central nervous system, particularly in cortical and spinal motor neurons and hippocampus (Murmu et al., 2011). Spastizin colocalizes with markers of endoplasmic reticulum, endosomes and mitochondria, suggesting a role in intracellular trafficking and/or mitochondrial functions (Hanein et al., 2008). Spastizin is ubiquitously expressed in all tissues and indicate a function in embryonic development (Hanein et al., 2008; Murmu et al., 2011).

Slabicki et al. (2010) identified a novel HSP-associated gene, KIAA0415/SPG48 and showed that helicase encoded by KIAA0415 interacts with SPG11 and SPG15. Since KIAA0415 protein functions in decreasing the frequency of homologous recombination during DNA double-strand break repair, this finding suggests a link between HSP and DNA repair (Slabicki et al., 2010).

Schicks et al. (2011) showed SPG15 as the causative gene in a consanguineous Juvenile Parkinsonism family, extending the phenotypic spectrum of SPG15. Considering SPG11-associated with Parkinsonism (Anheim et al. 2009) the overlap between SPG15 and SPG11 is further reinforced (Schicks et al., 2011).

1.2.5. SPG20

SPG20 is mappped to chromosome 13q12.3 and is associated with autosomal recessive complicated type HSP, namely Troyer syndrome (TRS) (Patel et al., 2002). TRS is observed due to a founder mutation in Old Order Amish families (Patel et al., 2002) and includes the symptoms, such as mild developmental delay, skeletal abnormalities (short stature), dysarthria and distal amyotrophy (Proukakis et al., 2004). The causative mutation in TRS patients in Amish population is a frameshift mutation (1110delA) however; a recent study reported two Omani HSP families bearing a novel 2bp deletion (c.364_365delAT) mutation in SPG20 gene (Manzini et al., 2010).

SPG20 codes for a protein called spartin which share sequence similarity with animal proteins SNX15, VPS4, Skd1 that regulate endosome morphology and funtion in endosome and protein trafficking (Patel et al., 2002). Additionally, spartin has a MIT domain that is closely related to MIT domain of spastin, indicating a possible functional link between two genes responsible for HSP (Ciccarelli et al., 2003). However, when phenotypical differences in SPG4 and SPG20-associated HSP are considered, it would be important to figure out differences in expression and localization profile of these genes (Burgunder and Hunziker, 2003).

Spartin is found to be a cytosolic and membrane-associated protein and interacting with Eps15 that is involved in endocytosis and the control of cell proliferation, further enhancing the possible function of spartin in endocytosis and vesicle trafficking (Bakowska et al., 2005). Moreover, Lu et al. (2006) reported that spartin associates with microtubules and localizes to mitochondria via its plant-related senescence domain in C-terminus, suggesting a role in microtubule-mediated trafficking of mitochondria. Further evidence came from Joshi and Bakowska (2011) reporting that spartin localizes to mitochondria and functions in maintaining mitochondrial Ca^{2+} homeostasis.

Another distribution study showed that two isoforms of spartin localizes to different subcellular parts such that 100 kDa isoform is present in the nucleus, as well as in the cytoskeleton and 85 kDa phosphorylated variant is present in the membrane-enriched parts (Robay et al., 2006). Additionally, Milewska et al. (2009) reported that spartin interacts with a nucleolar protein, nucleolin.

Bakowska et al. (2007) showed that spartin has a role in regulation of the epidermal growth factor receptor (EGFR), as well as Eps15, indicating involvement of spartin and Eps15 in intracellular trafficking of EGFR. In the same study, ~ 30–35% of endogenous spartin is found to be mono-ubiquitinated, providing evidence for its post-translational modification (Bakowska et al., 2007).

Two further studies implicated spartin in the regulation of lipid droplet (LD) turnover (Eastman et al., 2009; Hooper et al., 2010). However, how LD metabolism is related to HSP is not known. Furthermore, Tsang et al. (2009) demonstrated that spartin functions in inhibiting BMP signaling. Still other studies demonstrated that spartin is involved in

cytokinesis since it is recruited to the midbodies by the endosomal sorting complex required for transport protein Ist1 (ESCRT-III protein Ist1 - increased sodium tolerance 1) (Renvoise et al., 2010; Lind et al., 2011).

In the light of these studies that reported diverse localization and function for spartin, it can be suggested to be a multifunctional protein, explaining the complex phenotype observed in SPG20-associated HSP patients (Edwards et al., 2009; Milewska et al., 2009). Consistently, Milewska et al. (2009) determined 94 proteins that potentially interact with spartin via a proteomic approach of Tandem Affinity Purification (Fig 1.5).

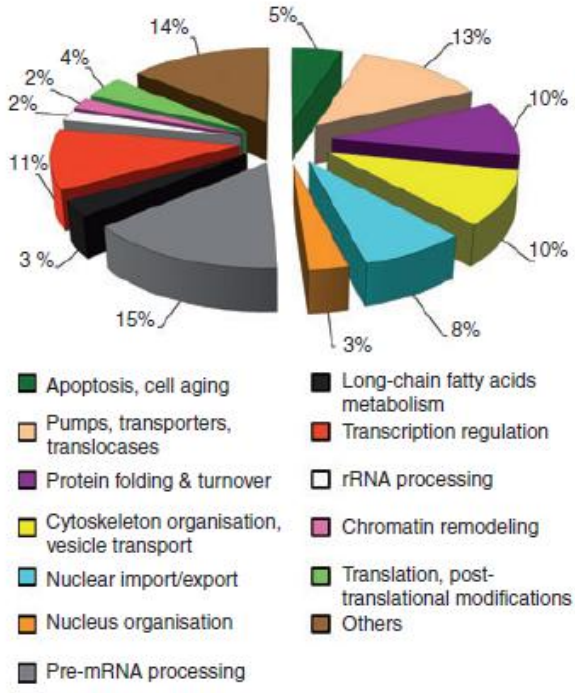


Figure 1.5. Pie chart representing 94 putative spartin-interacting proteins (adapted from Milewska et al., 2009).

1.2.6. SPG21

SPG21 located on chromosome 15q22.31, is associated with an adult onset ARHSP-TCC with dementia (Mast syndrome) and mutation has been detected among the Old Order Amish (Simpson et al., 2003). A base-pair insertion (601insA) is observed in Mast syndrome patients. SPG21 codes for protein maspardin (ACP33) which localizes to endosomal/*trans*-Golgi transportation vesicles, suggesting a function in protein transport (Simpson et al., 2003; Hanna and Blackstone, 2009).

Maspardin is found to be interacting with the aldehyde dehydrogenase 16A1 (ALDH16A1) that funcions in aldehyde metabolism. Since long-lived aldehydes are highly reactive, pathogenic mechanism leading to HSP may be this toxic effect of unmetabolized aldehydes (Hanna and Blackstone, 2009).

Maspardin bears a domain with high similarity to α/β hydrolases, however, absence of catalytic triad in this protein indicates that maspardin lacks enzymatic activity and may act as a mediator in protein–protein interactions (Simpson et al., 2003).

Soderblom et al. (2010) reported alterations in axon branching in cerebral cortical neurons cultured from SPG21$^{-/-}$ mouse model, showing that this model might be used to understand the maspardin function and how mutant forms of this protein lead to HSP.

The clinical features and the protein products of autosomal recessive HSP loci are summarized in Table 1.3. SPG5, SPG7, SPG11, SPG15, SPG20 and SPG21 are HSP loci in which causative gene have been identified and analyzed in this study.

Table 1.3. Autosomal recessive forms of HSP (modified from Salinas *et al.*, 2008; Stevanin *et al.*, 2008).

Locus	Chromosome region	Gene (Protein)	Function of the protein	Frequency of mutations	Clinical features
SPG5	8q12.3	*CYP7B1*	Cholesterol and neurosteroid metabolism	5 mutated families (10% by linkage)	Variable-onset pure HSP
SPG7	16q24.3	*SPG7* (paraplegin)	Mitochondrial ATPase	1%–4%	Variable onset, cerebellar signs, optic atrophy, neuropathy
SPG11	15q14	*KIAA1840* (spatacsin)	Unknown	21% (59% of ARHSP-TCC)	Childhood to early adult onset, thin corpus callosum, cognitive impairment, neuropathy
SPG14	3q27-q28	*Unknown*		1 family	Variable onset, motor neuropathy, mental retardation

Table 1.3. Autosomal recessive forms of HSP (modified from Salinas *et al.*, 2008; Stevanin *et al.*, 2008) (continued).

Locus	Chromosome region	Gene (Protein)	Function of the protein	Frequency of mutations	Clinical features
SPG15	14q24.1	*ZFYVE26* (spastizin)	Endosomal trafficking	15% in one study (10 families)	Kjellin syndrome: adolescent onset, pigmented retinopathy, cerebellar signs, mental retardation
SPG20	13q12.3	*KIAA0610* (spartin)	Microtubule interaction, endosomal trafficking	Amish founder	Troyer syndrome: childhood onset, amyotrophy, cerebellar signs, developmental delay
SPG21	15q21-q22	*ACP33* (maspardin)	Endosomal, trans-Golgi trafficking	Amish founder	Mast syndrome: early adult onset, thin corpus callosum, cognitive decline, extrapyramidal features, cerebellar signs
SPG23	1q24-q32	Unknown		1 family	Lison syndrome: childhood onset, pigmentary abnormalities, facial and skeletal dysmorphism, cognitive decline, tremor
SPG24	13q14	Unknown		1 family	Childhood-onset pure HSP, pseudobulbar signs
SPG25	6q23-24.1	Unknown		1 family	Adult onset, cataracts, prolapsed intervertebral discs
SPG26	12p11.1-12q14	Unknown		2 families	Adult onset, neuropathy and distal wasting, intellectual impairment
SPG27	10q22.1-q24.1	Unknown		2 families	Variable onset, cerebellar signs, neuropathy, mental retardation, microcephaly
SPG28	14q21.3-22.3	Unknown		1 family	Early-onset pure HSP
SPG30	2q37.3	Unknown		1 family	Adolescent-onset pure HSP, sensory neuropathy

Table 1.3. Autosomal recessive forms of HSP (modified from Salinas *et al.*, 2008; Stevanin *et al.*, 2008) (continued).

Locus	Chromosome region	Gene (Protein)	Function of the protein	Frequency of mutations	Clinical features
SPG32	14q12-q21	Unknown		1 family	Childhood onset, mental retardation, thin corpus callosum, pontine dysraphism
SPOAN	11q13	Unknown		1 family	
SPG35	16q21-q23	Unknown		1 family	Childhood onset, intellectual decline, seizures
SPG39	19p13	*PNPLA6*	Neuropathy target esterase	2 families	Childhood onset, marked distal wasting in all four limbs

1.3. X-linked HSP

X-linked HSP is associated with three loci: SPG1, SPG2 and SPG16. The clinical features and the protein products of autosomal recessive HSP loci are summarized in Table 1.4.

SPG1 is mapped to chromosome Xq28 and is associated with complicated HSP. Causative mutations are found in neural cell adhesion molecule L1 (L1CAM) gene. Other mutations in this gene can also lead to MASA syndrome (syndrome of mental retardation, aphasia, shuffling gait, adducted thumbs) or X-linked hydrocephalus (Jouet et al., 1994). L1CAM is a transmembrane glycoprotein which is expressed mainly in neurons and functions in cellular processes such as neuronal cell migration, myelination, axonal growth (Hortsch, 2000). L1 family protein members are composed of immunoglobulin (Ig)-protein domains, fibronectin type II domains, transmembrane and cytoplasmic region (Jouet et al., 1994).

SPG2 is located on chromosome Xq21-q22. Mutations in proteolipoprotein gene (PLP1) cause complicated HSP and Pelizaeus-Merzbacher disease. PLP1 and its isoform DM20 are highly expressed in myelin cells in central nervous system (Garbern et al.,

2007). Autopsy results of patients with null mutations of PLP1 showed length-dependent axonal loss (Garbern et al., 2002). Edgar et al. (2004) reported impairment of fast anterograde and retrograde transport in PLP null mice, suggesting a link of PLP1 with axonal trafficking.

The last X-linked HSP locus is SPG16, for which responsible gene is not identified, yet. Affected individuals have motor aphasia, mental retardation and sphincter disturbance (Steinmuller et al., 1997).

Table 1.4. X-linked forms of HSP (modified from Salinas *et al.*, 2008; Stevanin *et al.*, 2008).

Locus	Chromosome region	Gene (Protein)	Function of the protein	Frequency	Clinical features
SPG1	Xq28	L1 cell adhesion molecule (L1CAM)	Cell adhesion, neurite outgrowth, myelination	Over 100 familial cases	Mental retardation, TCC, adducted thumbs, hydrocephalus
SPG2	Xq21	Proteolipoprotein 1 (PLP)	Primary constituent of myelin	<100 familial cases	Quadriplegia, nystagmus, mental retardation, seizures, allelic to Pelizaeus-Merzbacher
SPG16	Xq11.2	Unknown		1 family	HSP with onset in infancy, aphasia, sphincter disturbance, mental retardation

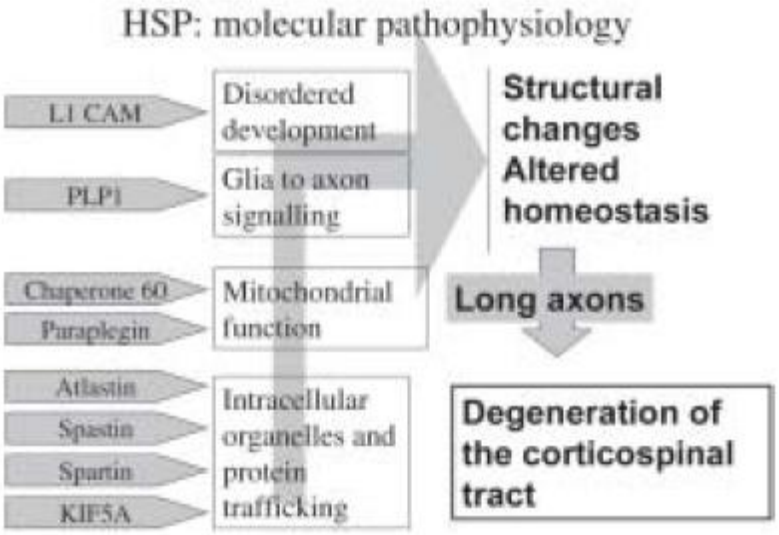


Figure 1.6. Summary of identified molecular mechanisms leading to degeneration of corticospinal tract in HSP (adapted from Burgunder et al., 2003).

2. AIM OF THE STUDY

In the scope of this study, our aim is to study the molecular basis of HSP in a cohort of 15 families with possible autosomal recessive inheritance. Although autosomal dominant HSP is more frequent in European countries, autosomal recessive forms predominate in inbred populations. Since consanguineous marriages are common, especially in some regions of Turkey, autosomal recessive spastic paraplegia (ARHSP) is almost 50% among our cohort.

More specifically, we aimed to perform haplotype analysis to test linkage to ARHSP loci for which the responsible genes have already been identified. These loci were SPG5, SPG7, SPG11, SPG15, SPG20 and SPG21. Therefore, the main purpose is to exclude these known genes in Turkish families that we believe will be helpful for identification of novel genes in future studies.

3. MATERIALS

3.1. Subjects

Peripheral blood samples of Turkish HSP patients and their family members were provided by the following neurology departments:, Istanbul University, Cerrahpaşa Medical Faculty, Istanbul Medical Faculty, Pamukkale University, Marmara University, Gazi University, and Department of Pediatric Neurology, Çukurova University. Informed consent was obtained from all family members and the study was approved by Ethics Review Committees of participating institution.

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 (Italy), unless stated otherwise in the text.

3.3. Buffers and Solutions

3.3.1. DNA Extraction from Peripheral Blood

Table 3.1. DNA extraction materials.

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)
Sodiumdodecylsulfate (SDS)	:	10 per cent SDS (w/v) (pH 7.2)
Proteinase K	:	20 mg/ml in H2O

Table 3.1. DNA extraction materials (continued).

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)

Table 3.2. Polymerase Chain Reaction materials.

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)
Dimethylsulfoxide (DMSO)	:	Stock solution (Fermentas, Lithuania)

3.3.3. Agarose Gel Electrophoresis

Table 3.3. Agarose Gel Electrophoresis materials.

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 (Basica LE) in 0.5 X 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 (Fermentas, Lithuania)

3.4. Fine Chemicals

3.4.1. Enzymes

Taq DNA Polymerase and the restriction enzyme *Msp*I were purchased from Fermentas (Lithuania). The restriction enzymes *Bfa*I, *Bsa*I, *Bsaj*I, *BsmA*I, *BspM*I, *Hind*III, *Hpy188*I, *Mbo*II, *Nsp*I, *Sac*I and *Sfc*I were obtained from New England Biolabs (UK). The restriction enzymes *Fai*I and *Set*I were purchased from SibEnzyme (USA).

3.4.2. Oligonucleotide Primers

The sequences of the primers used for amplification of SNP markers of SPG5, SPG7, SPG11, SPG15, SPG20 and SPG21 loci are given in Tables 3.4, 3.5, 3.6, 3.7, 3.8 and 3.9 respectively. The sequences of the primers to amplify exons of SPG5 and SPG7 are listed in the Tables 3.10 and 3.11, respectively. These primers were purchased from Iontek (Turkey).

Table 3.4. Sequences of the primers used for the amplification of SNP markers in SPG5 locus.

Marker	Primer (F/R*)	Primer Sequence (5'→3')	Size (bp)	Annealing Temp.(°C)
rs10808738	SNP5.1F	GCTCCTTAATGTTGGTCTTGG	158	59°C
	SNP5.1R	TCCATTCTGTAGGTTTTCTTTTGA		
rs10957321	SNP5.2F	CAAGCACTTGGGATGTGTGT	194	62°C
	SNP5.2R	CCTCTGAAGGCTCAGTGTCA		
rs7830315	SNP5.3F	ATGGATGTGTCATCAGCACC	197	59°C
	SNP5.3R	TTGAAAACATTTTGCACATCAA		
rs13252547	SNP5.4F	AAGTCCAAATGTGTGTAAATGTGTG	212	61°C
	SNP5.4R	TGCAGTTCAAACTCACAATGTTT		

* F: Forward primer, R: Reverse primer

Table 3.5. Sequences of the primers used for the amplification of SNP markers in SPG7 locus.

Marker	Primer (F/R*)	Primer Sequence (5'→3')	Size (bp)	Annealing Temp.(°C)
rs8046182	SNP7.1F	TTGTGATCTGCTCACCTTGG	172	61°C
	SNP7.1R	AATCTCAGCACTTTGGGAGG		
rs8060502	SNP7.2F	GGCCTCCATGAAGAGTGAGT	144	62°C
	SNP7.2R	CCCTGTTCTCCAGACATTGG		
rs7193472	SNP7.3F	CTAAGGGGTGGGAGCTCAG	169	59°C
	SNP7.3R	AGAGGGTGAAAGGACAAGGG		

* F: Forward primer, R: Reverse primer

Table 3.6. Sequences of the primers used for the amplification of SNP markers in SPG11 locus.

Marker	Primer (F/R*)	Primer Sequence (5'→3')	Size (bp)	Annealing Temp.(°C)
rs2555355	SNP11.1F	TACACCACCACACCTGGCTA	174	59°C
	SNP11.1R	CAATTTTGCAGGACCATTTTT		
rs3759871	SNP11.2F	CTCACCTGTCAAAACAAAGCAC	143	62°C
	SNP11.2R	CTTGGGAAGTGGAAAGGATG		

* F: Forward primer, R: Reverse primer

Table 3.7. Sequences of the primers used for the amplification of SNP markers in SPG15 locus.

Marker	Primer (F/R*)	Primer Sequence (5'→3')	Size (bp)	Annealing Temp.(°C)
rs1955468	SNP15.1F	GCACTTAGTATCATGCCTGGAA	187	62°C
	SNP15.1R	ATGACAAGCCACCAAAAGGA		
rs2295108	SNP15.2F	GGCTCCTGTTAGTGGACAATG	199	62°C
	SNP15.2R	GTACAGCCTCTGTCGGAAGG		
rs181576	SNP15.3F	TTGGAGAACAGGGCTAGCAT	180	62°C
	SNP15.3R	AAAGTATGGAAAACCCAGCC		

* F: Forward primer, R: Reverse primer

Table 3.8. Sequences of the primers used for the amplification of SNP markers in SPG20 locus.

Marker	Primer (F/R*)	Primer Sequence (5'→3')	Size (bp)	Annealing Temp.(°C)
rs9547229	SNP20.1F	GAGCCATGTAAATGTTCCCC	151	60°C
	SNP20.1R	GTGAACAAGGGAGCACGAG		
rs755051	SNP20.2F	GGCCTAGAAGCACAAAATGG	165	60°C
	SNP20.2R	AGGCAAACAGTTGACATGGA		
rs7335635	SNP20.3F	CATGGGACATACACCAACGA	164	62°C
	SNP20.3R	AAAGTGGAAGATTGTGAAAGGA		

* F: Forward primer, R: Reverse primer

Table 3.9. Sequences of the primers used for the amplification of SNP markers in SPG21 locus.

Marker	Primer (F/R*)	Primer Sequence (5'→3')	Size (bp)	Annealing Temp.(°C)
rs17229765	SNP21.1F	CAGGCTGTTTTCAGTGACCA	186	60°C
	SNP21.1R	TTGGGAAATTATTCATTATAAGCG		
rs4776658	SNP21.2F	ACAACGCTTCCTACCGCTCT	144	62°C
	SNP21.2R	CTTTTGAAGGAGGCTGGTGT		

* F: Forward primer, R: Reverse primer

Table 3.10. Sequences of the primers to amplify exons of CYP7B1 (SPG5) gene (sequnces obtained from Schüle et al., 2009a).

Exon	Primer (F/R*)	Primer Sequence (5'→3')	Size (bp)	Annealing Temp.(°C)
Exon 1	SPG5.1F	GAACTTTTGTCATTCAGCCT	409	55
	SPG5.1R	TGTCTGCACTGGAAATCATG		
Exon 2	SPG5.2F	AGCAGTGCAATCCATGCAGT	463	55
	SPG5.2R	GGTTCCCTAAGTACCATGAAG		

Table 3.10. Sequences of the primers to amplify exons of CYP7B1 (SPG5) gene (sequnces obtained from Schüle et al., 2009a) (continued).

Exon	Primer (F/R*)	Primer Sequence (5'→3')	Size (bp)	Annealing Temp.(°C)
Exon 3	SPG5.3F	CATGTAGTGTACCTTCGAATG	837	62
	SPG5.3R	TTCAAGGTCGCCATTTTGTC		
Exon 4	SPG5.4F	GGTTCTCATTAGCATGCACTG	437	58
	SPG5.4R	AATTAGGCAGCTGTGTCCTC		
Exon 5	SPG5.5F	GAGAGCAGTTTTCAGGACCA	500	61
	SPG5.5R	GGCGCAATGTCCACTTCATT		
Exon 6	SPG5.6F	AGCAACTTTGTGGACTTGAAC	501	30
	SPG5.6R	CTGGACTGATATCAGATCAAAT		

* F: Forward primer, R: Reverse primer

Table 3.11. Sequences of the primers to amplify exons of paraplegin (SPG7) gene (Wilkinson et al., 2004).

Exon	Primer (F/R*)	Primer Sequence (5'→3')	Size (bp)	Annealing Temp.(°C)
Exon 1	SPG7.1F	ATCACGCAGGCGCGGCTTTCAG	270	60
	SPG7.1R	CTGGGCCTTACAGAGCAGA		
Exon 2	SPG7.2F	AGTCTGCATTGCTTTGGTACT	228	57
	SPG7.2R	TAGCTGAGGCGATAAGTGTG		
Exon 3	SPG7.3F	GGAGTACACTGTTGTCCTGT	226	55
	SPG7.3R	ACAGAAATGTAAAGACATCCAG		
Exon 4A	SPG7.4AF	AAGCTCTGGATGTCGCCCGT	193	57
	SPG7.4AR	AGGAAATGCTGCCTCCGCTG		
Exon 4B	SPG7.4BF	GCGGTTGTCATGAGCCTCCT	241	57
	SPG7.4BR	CTCACTCTCACAGGCTGCCA		
Exon 5	SPG7.5F	GACTGTAGGGTTGCTCGTCT	260	55
	SPG7.5R	CAGATTACAAAGCCAAGTTAGG		
Exon 6	SPG7.6F	TTGGAAGCCTGCGTCTGTCA	225	57
	SPG7.6R	GTATTCAGCAAACACAAACCAG		

* F: Forward primer, R: Reverse primer

Table 3.11. Sequences of the primers to amplify exons of paraplegin (SPG7) gene (Wilkinson et al., 2004) (continued).

Exon	Primer (F/R*)	Primer Sequence (5'→3')	Size (bp)	Annealing Temp.(°C)
Exon 7	SPG7.7F	CTGGCATCGTGCTGCTGATT	257	57
	SPG7.7R	CCCTTCTGGGAGAGGAGGA		
Exon 8	SPG7.8F	AGTGTTGCATTGTCTGCTGC	252	57
	SPG7.8R	ATGTGTGAAAGGAGCCAGGT		
Exon 9A	SPG7.9AF	CCTTGGTGTAGAACTTTGTCT	221	55
	SPG7.9AR	TGTTGGAGAAGCCGGACATG		
Exon 9B	SPG7.9BF	GCATCGTCTACATCGATGAG	187	55
	SPG7.9BR	CCTGTTCTGAAAGACATCGG		
Exon 10	SPG7.10F	TCCCTCCTGTGTCCTGAAGG	283	57
	SPG7.10R	CCAGACCACTCAGAGCGAGT		
Exon 11	SPG7.11F	ACCTGTGGCAGTAACTAGGT	211	57
	SPG7.11R	GCCTTGATGCTGTTTGCGCA		
Exon 12	SPG7.12F	CTCTTAAGCCCTGATAGCAG	252	55
	SPG7.12R	TCACCTCTCAATACCTGCCT		
Exon 13	SPG7.13F	GTCTCGAACTCCTGTCCTCA	300	60
	SPG7.13R	AGTCAGCTACAGACACAGGC		
Exon 14	SPG7.14F	ACGGAGACCTCTTAGTCCCA	321	55
	SPG7.14R	CATGGCATGCACTGGAACAG		
Exon 15	SPG7.15F	ACTGCTCTGCGCCTGCAGT	294	57
	SPG7.15R	CCTTGTGTGGTAGACCCA		
Exon 16	SPG7.16F	TCTGTGCTTTGGTGCTGGAG	206	57
	SPG7.16R	ACCGTGGGTGCTGTGTGGA		
Exon 17	SPG7.17F	ACATGCATATGCCTGTTCTTT	312	57
	SPG7.17R	CTCAGCTGAAAAGCAACTCAG		

* 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. DNA Sequencing Chemicals

DTCS Quick start master mix, 3M Na-Ac, 3M Na-EDTA, glycogen were purchased from Beckman Coulter (USA) and pure H_2O was purchased from Sigma-Aldrich (Germany).

3.4.5. Other Fine Chemicals

High Pure PCR Purification Kit was purchased from Roche (USA).

3.5. Equipment

Automated DNA Sequencing was performed using the GenomeLab™ GeXP Genetic Analysis System by DNA core facility in Boğaziçi University. The other equipments used in the study are listed in Table 3.12.

Table 3.12. Equipments used in this study.

Autoclaves	:	ASB270NT (Astell, UK)
Balances	:	Electronic Balance Model VA124 (Gec Avery, UK) Electronic Balance Model CC081 (Gec Avery, UK)
Centrifuges	:	Centrifuge 5415C (Eppendorf, 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	:	GelDoc Documentation System (Bio-Rad, USA)

Table 3.12. Equipments used in this study (continued).

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	:	Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA)
Thermocyclers	:	iCycler (Bio-Rad, USA) MyCycler (Bio-Rad, USA)
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 were taken into tubes containing K_3EDTA to prevent coagulation. Tubes were kept at 4 ˚C until DNA extraction. Thirty ml red blood cell (RBC) lysis buffer is added to the blood samples that were transferred to sterile 50 ml Falcon tubes. After vortexing, the blood and buffer mix were left at 4 ˚C for 25 minutes to lyze the erythrocyte membranes, by vortexing in 10^{th} and 20^{th} minutes. For collecting the leukocyte nuclei, the solution was centrifuged at 5000 revolution per minute (rpm), at 4 ˚C for 10 min. The supernatant was discarded and 10 ml RBC lysis buffer was added on the pellet to resuspend it. After vortexing, the resuspension was centrifuged at 5000 rpm, at 4 ˚C for an additional 10 minutes. The supernatant was discarded and the nuclei pellet was resuspended in three ml nuclei lysis buffer by vortexing. Storage of nuclei at -20 ^{0}C or -70^{0}C was possible at this stage. Then 30 µl Proteinase K (Section 3.4.1) and 50 µl of 10% SDS (w/v) were added to degrade the cellular proteins and the samples were incubated at 37 ºC overnight for proper functioning of enzyme. Afterwards, 10ml of 2.5 M NaCl was added and the mixture was shaken vigorously. The samples were centrifuged at 7500 rpm at room temperature for 30 min. The supernatant was taken into a new 50 ml Falcon tube and two volumes of -20^{0}C-cold absolute ethanol were added to precipitate the DNA. The DNA was fished out and transferred to a sterile 1.5 ml eppendorf tube. After air drying of the DNA pellet to evaporate ethanol, 200-500 µl of Tris-EDTA (TE) buffer (Section 3.5.1) was added and the samples were left overnight at room temperature to dissolve completely.

4.2. Quantitative Analysis of Extracted DNA

The concentration of the genomic DNA was measured by using the Nanodrop ND-1000 spectrophotometer, at 260 nm. The measurement was done based on the fact that 50 µg of double stranded DNA has an absorbance of 1.0 at 260 nm. The purity of the DNA was checked by A_{260}/A_{280} ratio which should be between 1.8 -2.

4.3. Haplotpye Analysis

Haplotype analysis was performed for 15 (Table 4.1) families to test linkage to SPG5, SPG7, SPG11, SPG15, SPG20 and SPG21 loci by restriction endonuclease analysis of SNP markers selected for related HSP loci. The clinical data for the index cases used in the study were given in Table 4.1.

Table 4.1. Clinical data of the index patients.

Family Number	Consanguinity	Affected members	Age of onset (years)	Phenotype
P463	First cousin	2 sisters	19	Distal weakness, TCC*, cortical atrophy, pes cavus, mental impairment, spasticity at bilateral lower extremities
P627	Non-consanguineous	2 sisters	>20	Not mentioned
H6	Consanguineous	2 siblings	11	Pure HSP
H28	Non-consanguineous	3 siblings	>20	Pure HSP
H29	Consanguineous	3 siblings	12-13	TCC*, sphincter muscle disturbances, cerebellar atrophy, decreased vibration, pigmentation abnormality, cerebellar signs
H36	From the same village	2 brothers	27	Peripheral neuropathy
H38	First cousin	3 siblings	>40	TCC*

Table 4.1. Clinical data of the index patients (continued).

Family Number	Consanguinity	Affected members	Age of onset (years)	Phenotype
H45	Consanguineous	3 siblings	26	Peripheral neuropathy, dementia, mental retardation
H46	First cousin	1 sibling	33	Pure , scoliosis, diminished sensation in left leg
H49	First cousin	4 siblings, 1 nephew	12	TCC*
H50	Consanguineous	2 sisters	14	TCC*, pes cavus
H52	Consanguineous	3 siblings	17	Pure HSP
H53	First cousin	2 brothers	18	Pure, pes cavus, dysarthria
H55	First cousin	3 siblings	32	Complicated, epileptic seizures, pes cavus, scoliosis

*TCC: thin corpus callosum

4.3.1. Determination of Single Nucleotide Polymorphism (SNP) markers

SNP markers for each locus were chosen based on the assumption that one SNP from each linkage disequilibrium (LD) block represents other SNPs in that block. Therefore, one SNP that corresponds to a restriction endonuclase site is chosen for each LD block. The SNP data were downloaded from web-site of International HapMap Project (http://hapmap.ncbi.nlm.nih.gov/). Since database offers only four population-related options, database created according to SNP variation in CEU: CEPH (Utah residents with ancestry from northern and western Europe) population was used. The downloaded data was then utilized in Haploview 4.1 program, which shows the SNPs on LD blocks and minor allele frequency (MAF) of each SNP. Since MAF gives information about the frequency with which less common allele occurs in a given population, MAF value around 0.5 indicate a SNP with high heterozygote frequency. Therefore, SNPs were selected

depending on two criteria: one, MAF of the SNP is at least 0.45; two, SNP is located on a restriction endonuclease site (Table 4.2).

Table 4.2. Selected SNPs and their positions on the chromosome.

Locus	SNP rs# cluster ID	Chromosomal position	Function
SPG5 (6 exons)	rs10808738	65544125	Intron 1
	rs10957321	65605878	Intron 1
	rs7830315	65608361	Intron 1
	rs13252547	65691703	Intron 4
SPG7 (17 exons)	rs8046182	89581216	Intron 3
	rs8060502	89589408	Intron 4
	rs7193472	89605495	Intron 9
SPG11 (40 exons)	rs2555355	44878413	Intron 7
	rs3759871	44943757	Non-pathogenic missense (exon 31)
SG15 (41 exons)	rs1955468	68237499	Intron 11
	rs2295108	68250622	Intron 19
	rs181576	68273430	Intron 33
SPG20 (8 exons)	rs9547229	36884923	Intron 1
	rs755051	36897233	Intron 1
	rs7335635	36916985	Intron 3
SPG21 (8 exons)	rs17229765	65258834	Intron 1
	rs4776658	65272664	Intron 3

CYP7B1 (SPG5) is located on a 202820-bp-site (65508529 – 65711348) on chromosome 8q12.3. Distances between the SNP markers chosen are 61753, 2483 and 83342, respectively. Size of the paraplegin gene (SPG7) is 49370 bp (89574805 - 89624174) on chromosome 16q24.3. SNP markers are located with distances 8192bp and 16087 bp. Spatacsin (SPG11) is mapped to a 100982-bp-region (44854894- 44955876) on chromosome 15. Distance between SNP markers are 65344 bp. SPG15 is located to a 77070 bp-long region on chromosome 14q24.1 (68213237- 68283306). 13123 bp and 22808 bp are the distances between SNP markers analyzed for SPG15 locus. SPG20 is mapped to chromosome 13q12.3 on a 68543 bp-region (36875775- 36944317). Distances

between SNP markers are 12310 bp and 19752 bp. SPG21 is located to a 26889 bp-site (65255363-65282251) on chromosome 15q21-q22. 13830 bp is the distance between two SNP markers.

4.3.2. Polymerase Chain Reaction (PCR)

The primers for the chromosomal regions including SNPs were designed by using the web-based software; Primer 3 (Subramaniam, 1998). (Table 3.4, 3.5, 3.6, 3.7, 3.8 and 3.9).

PCR reactions were performed in a volume of 25 µl containing 100 ng DNA, 2.5 µl of 10X polymerase buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 mM of each primer and 1U of *Taq* polymerase (Fermentas). The PCR program on Icycler^{TM} (BioRad) thermal cycler was as follows: an initial denaturation step at 95ºC for 5 min, followed by 35 cycles of 30 s at 95ºC, 30 s at annealing temperature, 30 s at 72ºC, a final elongation at 72°C for 5 min, and cooling at 15°C for 1 min. PCR products (5µl) were size fractionated on 2% (w/v) agarose gels to check for correct amplicon size.

4.3.3. Restriction Endonuclease Analysis

The amplified PCR products were digested with the appropriate restriction endonucleases (Table 4.3). Base variations either produce or abolish a restriction enzyme site so that, it is possible to determine which base is present in each allele of an individual. Ten µl of PCR product was digested with 3U of restriction enzyme and 1,5 µl of buffer, provided by the company, in a total volume of 15 µl. The samples were incubated at optimum temperature for each restriction enzyme. The digestion products were run on 2% agarose gel and stained by ethidium bromide to visualize DNA. Haplotypes were analyzed using EasyLINKAGE 5.08 software. FastLink 4.1 and SuperLink 1.6 – Two-Point Parametric Linkage Analysis softwares were run on EasyLINKAGE to calculate LOD scores. FastSLink 2.51 – Two-Point Pedigree Simulation Analysis software was run on EasyLINKAGE program to calculate possible maximum LOD score. Pedigrees constructed using SmartDraw software.

Table 4.3. Restriction enzymes used to digest amplified SNP fragments.

SNP rs# cluster ID	Restriction Enzyme	Reaction Temperature (°C)
rs10808738	*BsmA*I	55
rs10957321	*BspM*I	37
rs7830315	*Nsp*I	37
rs13252547	*Hpy188*I	37
rs8046182	*Fai*I	50
rs8060502	*Bsa*I	50
rs7193472	*Bfa*I	37
rs2555355	*Msp*I	37
rs3759871	*Mbo*II	37
rs1955468	*Sfc*I	37
rs2295108	*Alu*I	37
rs181576	*Sac*I	37
rs9547229	*Bsaj*I	60
rs755051	*Bsaj*I	60
rs7335635	*Set*I	55
rs17229765	*Hind*III	37
rs4776658	*Alu*I	37

4.4. DNA Sequence Analysis

PCR products were purified using the High Pure PCR purification kit (Roche). After purification, a second PCR reaction was performed to prepare the sample for sequencing. These PCR reactions were performed in a volume of 10 µl containing 4 µl Quickstart Master Mix (Beckman Coulter), 4.5 µl High Pure H_2O (Sigma-Aldrich), 5 pmol forward or reverse primer. Amount of PCR products used in these reactions were determined according to manufacturer's instructions. The PCR program on Icycler™ (BioRad) thermal cycler was as follows: 30 cycles of 20 s at 96ºC, 20 s at 50 ºC and 4 min at 60ºC. The products of second PCR reaction were cleaned up with ethanol precipitation and then, sequenced with GenomeLab™ GeXP Genetic Analysis System in Boğaziçi University.

The sequences were analyzed and aligned using Chromas Lite software and the ClustalW2 internet tool (Chenna *et al.*, 2003).

5. RESULTS

The molecular basis of Hereditary Spastic Paraplegia was investigated in 15 families with possible autosomal recessive inheritance. Fourteen of the families had at least two affected individuals and in 13, consanguinity was reported among parents. Only one of the families has single affected individual with consanguineous parents. In two of the families, parents were not consanguineous but there were at least two affected individuals.

5.1. Haplotype Analysis

Haplotype analysis for SPG5, SPG7, SPG11, SPG15, SPG20 and SPG21 loci were performed in our families. The responsible genes have already been previously identified for these loci. For each family, patients were analyzed, first. If linkage to analyzed locus cannot be excluded, then unaffected individuals are analyzed.

5.1.1. Haplotype Analysis of SPG5 Locus

In fourteen families, linkage to SPG5 locus was excluded (Figure 5.1). 26 patients were analyzed for these families, in total. The haplotypes of the family members that were excluded for linkage were given in the Appendix A part.

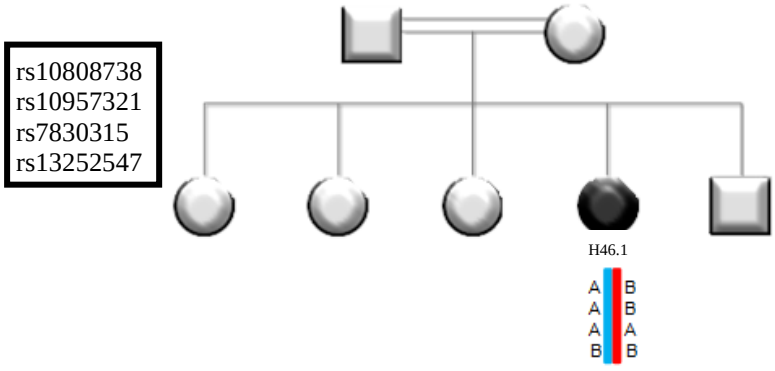


Figure 5.1. Haplotype analysis for family H46, for which linkage to SPG5 locus was excluded. The box on the left shows the order of the SNP markers used for SPG5 locus.

The four markers analyzed for SPG5 locus were non-informative for family H49 because two unaffected and four affected family members were found to be homozygous for these markers (Figure 5.2).

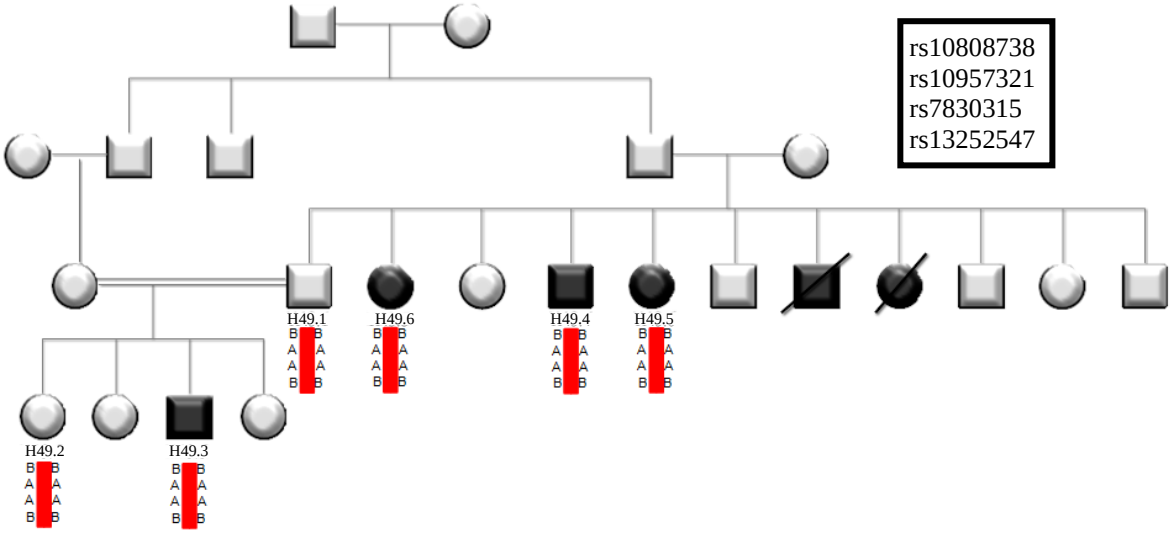


Figure 5.2 Haplotype analysis for family H49, for which the markers were non-informative for SPG5 locus. The box on the right shows the order of the SNP markers used for this locus.

Since the markers are non-informative, sequencing of six exons of the CYP7B1 gene was performed. Sequence alterations were not identified in the patient H49.5, for all exons (Figure 5.3 and Appendix I). After sequencing, linkage to SPG5 locus was also excluded in family H49.

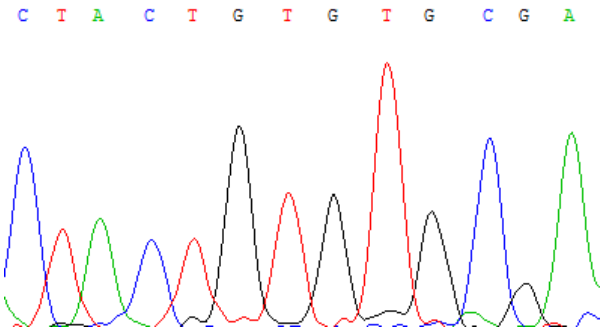


Figure 5.3. Partial chromatogram displaying (95th – 107th bases in the coding sequence) exon five of CYP7B1 gene in patient H49.5 in the sense strand.

5.1.2. Haplotype Analysis of SPG7 Locus

In ten of the families, linkage to SPG7 locus was excluded (Figure 5.3, 5.4 and Appendix B). For families H36, H46, H50, H52, and H55, the nature of the polymorphism could not be determined for at least one SNP marker, giving inconclusive results for these families. 33 patients and two unaffected individuals were analyzed for this part of the study.

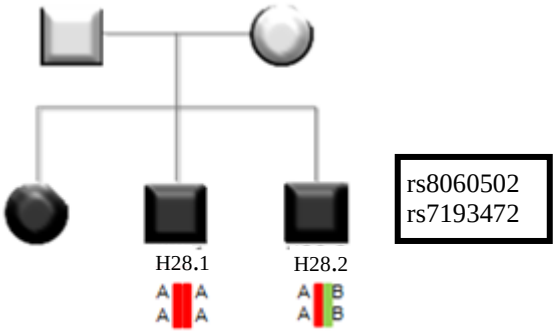


Figure 5.4. Haplotype of the family H28 for the markers spanning the SPG7 locus. Linkage to SPG7 is excluded for this family. The box on the right shows the order of the SNP markers used for this locus.

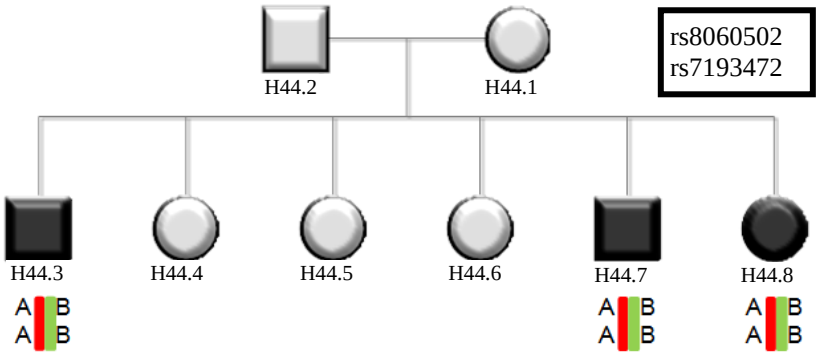


Figure 5.5. Haplotype analysis for family H44 for the markers spanning the SPG7 locus. Linkage to SPG7 is excluded for this family. Different colored bars show different haplotypes. The box on the right shows the order of the SNP markers used for SPG11 locus.

5.1.3. Haplotype Analysis of SPG11 Locus

In ten of the families, linkage to SPG11 locus was excluded (Appendix C). 26 patients and six unaffected individuals were analyzed for these ten families. After analyzing three affected and four unaffected individuals for family H45, a SPG11 haplotype segregating with the disease for two markers defining the SPG11 locus was found (Figure 5.5). Both FastLink 4.1 and SuperLink 1.6 softwares run on EasyLINKAGE 5.08 software calculated LOD score as 1.1767 (Figure 5.6). FastSLink 2.51 software gave the possible maximum LOD score as 2.05 (Figure 5.7 and Appendix G).

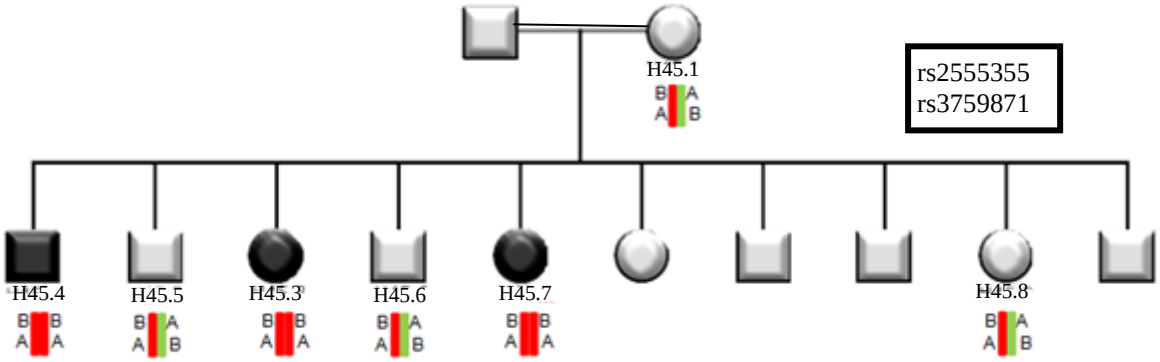


Figure 5.6. Haplotype analysis for family H45 for the markers spanning the SPG11 locus. Different colored bars show different haplotypes. The box on the right shows the order of the SNP markers used for SPG11 locus.

Marker	CHR	cM	LOD
1.rs2555355	15	44.00	1.1767
2.rs3759871	15	45.00	1.1767

Figure 5.7. LOD score results for family H45 calculated by FastLink 4.1 and SuperLink 1.6 run on EasyLINKAGE program.

Average Maximum Lod Scores based on quadratic interpolation

Pedigree	Average	StdDev	Min	Max
1	1.068304	0.546704	0.000000	2.053162
Study	1.068304	0.546704	0.000000	2.053162

Figure 5.8. Maximum LOD score results for family H45 calculated by FastSLink 2.51 run on EasyLINKAGE program.

In two families, P463 and P627, patients were found to be homozygous for the two markers analyzed for this locus (Figure 5.8). However, presence of linkage could not be investigated further since DNA samples from other family members were not available.

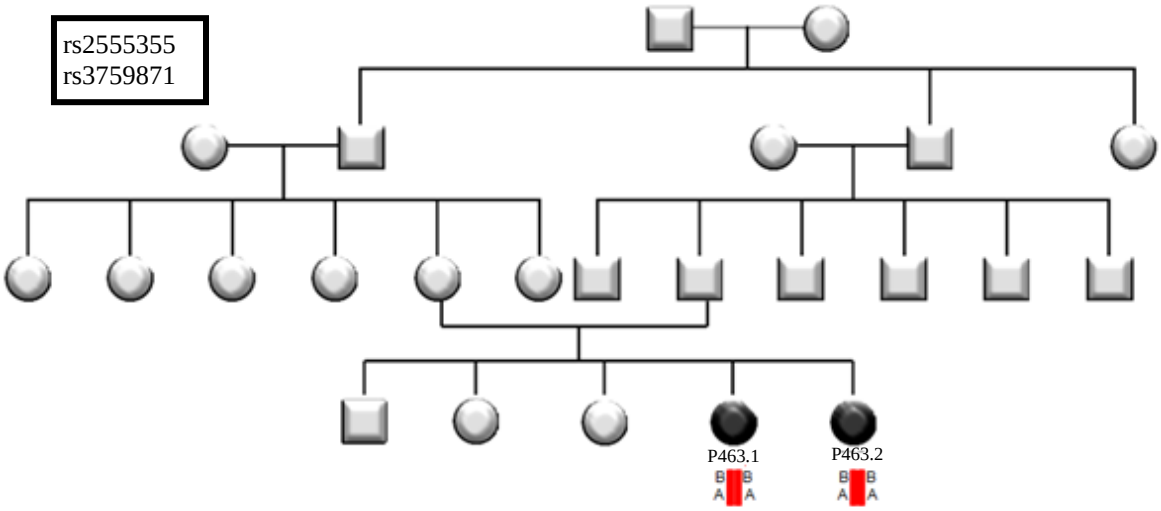


Figure 5.9. Haplotype analysis for family P463, for which the markers gave inconclusive results for SPG11 locus. The box on the left shows the order of the SNP markers used for this locus.

The markers analyzed for SPG11 were non-informative for families H36 and H50 (Figure 5.9). All individuals analyzed from these families were found to be homozygous for these markers.

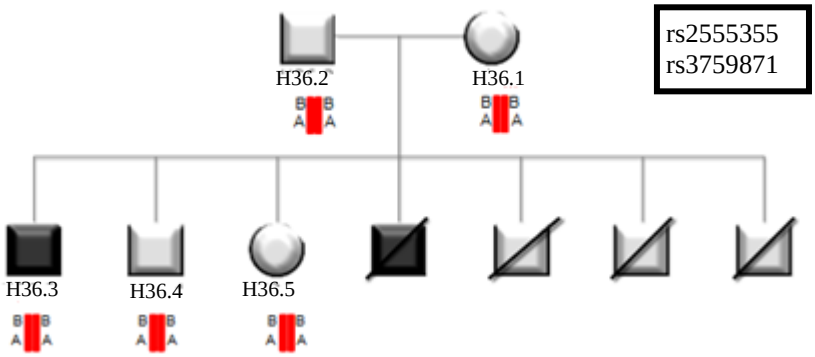


Figure 5.10. Haplotype analysis for family H36, for which the markers were non-informative for SPG11 locus. The box on the right shows the order of the SNP markers used for this locus.

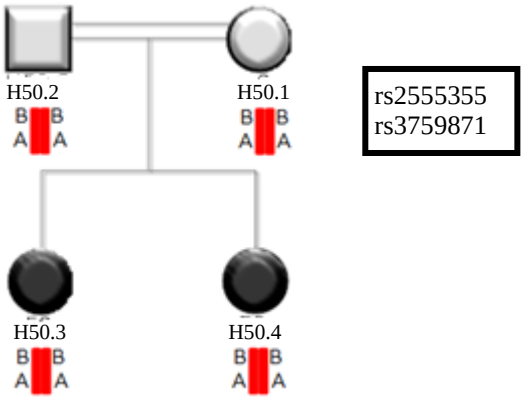


Figure 5.11. Haplotype analysis for family H50, for which the markers were non-informative for SPG11 locus. The box on the right shows the order of the SNP markers used for this locus.

5.1.4. Haplotype Analysis of SPG15 Locus

In family H29, SPG15 haplotype was segregating with the disease for the two markers defining the SPG15 interval (Figure 5.11). The other marker (rs1955468) used for this locus was non-informative for this family, as each individual was homozygous. Two affected and three unaffected individuals were analyzed for this family. SuperLink 1.6 software run on EasyLINKAGE 5.08 software calculated LOD score as 1.010 (Figure 5.12). FastSLink 2.51 software gave the possible maximum LOD score as 1.204 (Figure 5.13 and Appendix G).

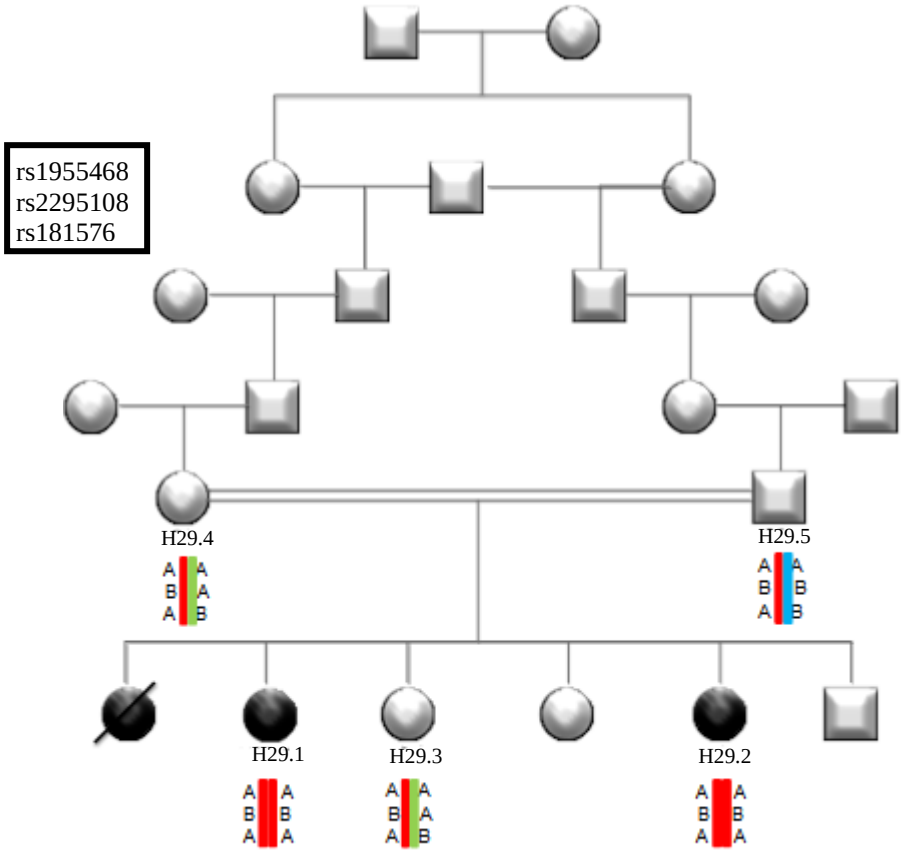


Figure 5.12. Haplotype analysis for family H29 for the markers spanning the SPG15 locus. Different colored bars show different haplotypes. The box on the left shows the order of the SNP markers used for SPG15 locus.

Marker	CHR	cM	LOD
1.rs181576	14	81.00	1.0109
2.rs2295108	14	80.00	0.7095

Figure 5.13. LOD score results for family H29 calculated by SuperLink 1.6.

```
---------------------------------------------------------------
 Average Maximum Lod Scores based on quadratic interpolation
---------------------------------------------------------------
Pedigree  Average        StdDev         Min            Max
     1       0.489640       0.263255       0.000000       1.204096

Study        0.489640       0.263255       0.000000       1.204096
---------------------------------------------------------------
```

Figure 5.14. Maximum LOD score results for family H29 calculated by FastSLink 2.51.

For one family, P627, the one affected individual was found to be homozygous for the three markers analyzed (Figure 5.14). However, we could not obtain DNA samples from other family members and presence of linkage remained uncertain.

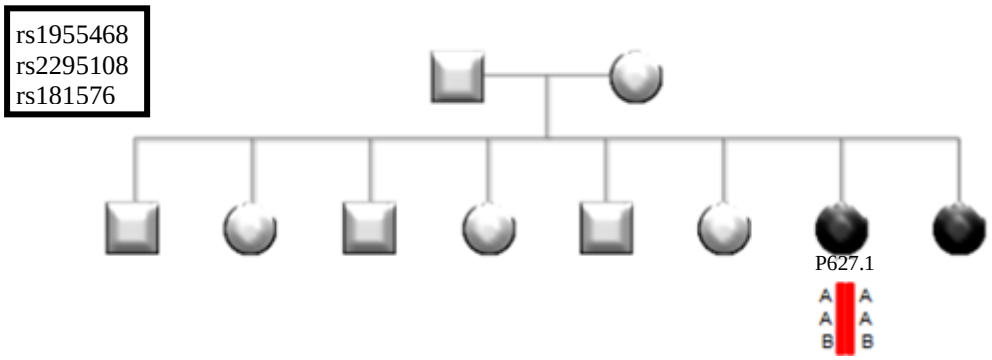


Figure 5.15. Haplotype analysis for family P627 for the markers spanning the SPG15 locus. Different colored bars show different haplotypes. The box on the left shows the order of the SNP markers used for SPG15 locus.

In thirteen of the families, linkage to SPG15 locus was excluded. For these families, 28 affected and four unaffected individuals were analyzed (Figure 5.15 and Appendix D).

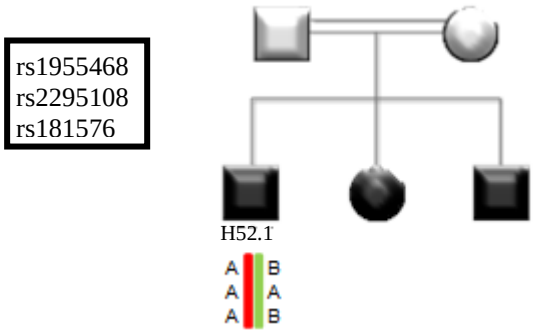


Figure 5.16. Haplotype of the family H52 for the markers spanning the SPG15 locus. The box on the left shows the order of the SNP markers used for this locus. Linakge to SPG 15 was excluded for this family.

5.1.5. Haplotype Analysis of SPG20 Locus

In family H6, two affected individuals were found to be homozygous for two of three markers analyzed for SPG20 locus (rs9547229, rs755051). The nature of the

polymorphism could not be identified for the other SNP marker (rs7335635). Thus, the markers gave inconclusive results (Figure 5.16).

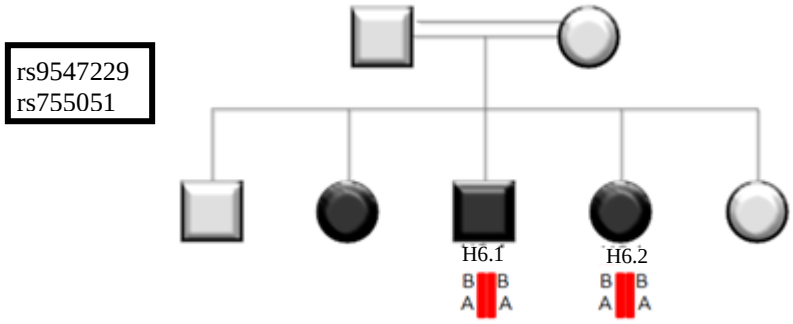


Figure 5.17. Haplotype analysis for family H6 for two of the markers spanning the SPG20 locus. Different colored bars show different haplotypes. The box on the left shows the order of the SNP markers used for SPG20 locus.

In fourteen of the families, linkage to SPG20 locus was excluded (Figure 5.17 and Appendix E). 27 patients and three unaffected individuals were analyzed for these families.

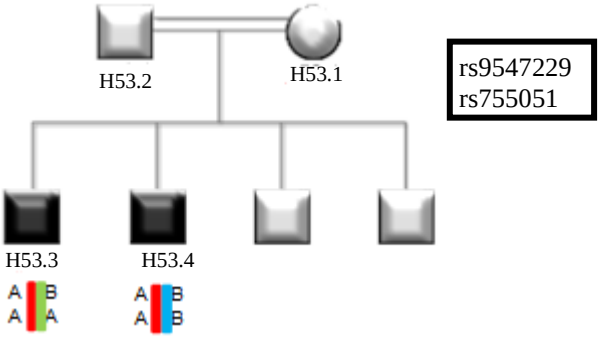


Figure 5.18. Haplotype of the family H53 for the markers spanning the SPG20 locus. The box on the right shows the order of the SNP markers used for this locus.

5.1.6. Haplotype Analysis of SPG21 Locus

In fourteen of the families, linkage to SPG21 locus was excluded. 24 affected and three unaffected individuals were analyzed for these families (Figure 5.18 and Appendix F).

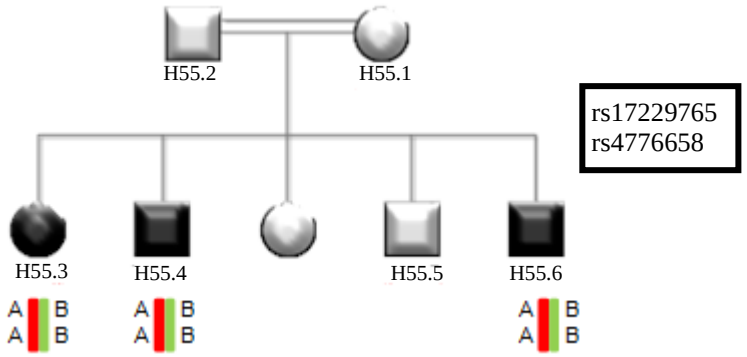


Figure 5.19. Haplotype of the family H55 for the markers spanning the SPG21 locus. The box on the right shows the order of the SNP markers used for this locus. Linkage to SPG21 was excluded in this family.

For family H38, all of the family members (three affected, five unaffected) were found to be homozygous for two markers. Thus, the markers analyzed were non-informative for this family (Figure 5.19)

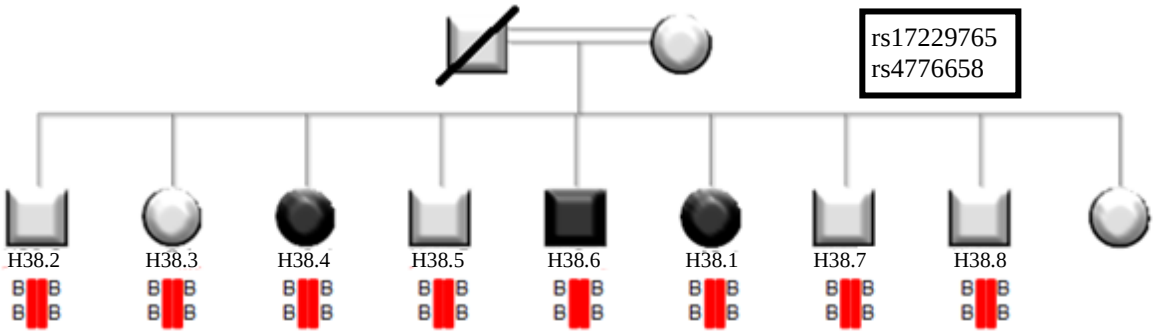


Figure 5.20. Haplotype analysis for family H38, for which the markers were non-informative for SPG21 locus. The box on the right shows the order of the SNP markers used for this locus.

Table 5.1. Summary of the results for linkage analysis of ARHSP loci.

	SPG5	SPG7	SPG11	SPG15	SPG20	SPG21
P463	Excluded	Excluded	Homozygous for patient	Excluded	Excluded	Excluded
P627	Excluded	Excluded	Homozygous for patient	Homozygous for patient	Excluded	Excluded
H6	Excluded	Excluded	Excluded	Excluded	Inconclusive	Excluded
H28	Excluded	Excluded	Excluded	Excluded	Excluded	Excluded
H29	Excluded	Excluded	Excluded	linked	Excluded	Excluded
H36	Excluded	Inconclusive	Non-informative	Excluded	Excluded	Excluded
H38	Excluded	Excluded	Excluded	Excluded	Excluded	Non-informative
H44	Excluded	Excluded	Excluded	Excluded	Excluded	Excluded
H45	Excluded	Excluded	linked	Excluded	Excluded	Excluded
H46	Excluded	Inconclusive	Excluded	Excluded	Excluded	Excluded
H49	Non-informative	Excluded	Excluded	Excluded	Excluded	Excluded
H50	Excluded	Inconclusive	Non-informative	Excluded	Excluded	Excluded
H52	Excluded	Inconclusive	Excluded	Excluded	Excluded	Excluded
H53	Excluded	Excluded	Excluded	Excluded	Excluded	Excluded
H55	Excluded	Inconclusive	Excluded	Excluded	Excluded	Excluded

6. DISCUSSION

In this study, SPG loci associated with autosomal recessive hereditary spastic paraplegia were analyzed to test linkage in 15 HSP families. The aim of the thesis was to exclude the known loci, thus, the genes responsible for the analyzed AR-HSP loci have already been identified. Among the families analyzed six of them had pure HSP while others had a complicated phenotype. Four families with complicated HSP had autosomal recessive inheritance with thin corpus callosum (ARHSP-TCC). Parents were consanguineous in thirteen families. Age of onset in our cohort varies within a range of 2-40 years.

Although autosomal dominant HSP is more common in western countries, frequency of autosomal recessive HSP is higher in inbred populations. In accordance with this observation, AR-HSP is observed more frequently in our population due to high rate of consanguineous marriages. Pedigree analysis among the HSP families referred to our laboratory for genetic analysis revealed approximately 50% ARHSP cases in the cohort.

6.1. Haplotype Analysis

The autosomal recessive HSP loci for which the responsible genes have been identified are SPG5, SPG7, SPG11, SPG15, SPG20 and SPG21. SPG48 gene has been identified after the project has started and was not included in this study. Although some clinical symptoms may help to associate the disease with a certain subtype of HSP, a clear distinction is not always possible because age of onset and disease severity vary not only between families but also within families. Therefore, linkage to all these loci was tested for all our families, regardless of their phenotypic features.

6.1.1. Haplotype Analysis of SPG5 Locus

SPG5 is generally associated with pure HSP (Bouslam et al., 2005) and only after long disease durations, cerebellar signs can be observed. On the hand, in a minority of

cases, some additional symptoms representing complicated phenotype are observed (Klebe et al, 2007). Age of onset varies from four to 47 years in SPG5-associated HSP. Six of our families had pure HSP but none of them were found to be linked to this locus. Linkage to SPG5 locus was excluded also for other families with complicated HSP. When the frequency of SPG5-associated HSP is considered (10%), absence of linkage can be expected, in our 15-family-cohort.

Individuals analyzed in H49 family were found to be homozygous for SPG5 markers, giving an inconclusive result for this family. Although complicated phenotype of the patients in this family (ARHSP-TCC) is not consistent with the general phenotype of the SPG5-related HSP, we sequenced the six exons of CYP7B1 gene in one of the affected individuals (H49.5). Expectedly, SPG5 mutation was not observed in this patient. Therefore, all families were excluded for SPG5 locus.

6.1.2. Haplotype Analysis of SPG7 Locus

SPG7-associated HSP patients may have either pure or complicated phenotype. Additional symptoms such as cerebellar signs, optic atrophy, and neuropathy can be observed and age of onset is variable (Wilkinson et al., 2004). Mutations in paraplegin cause 1-4% of ARHSP cases.

In 10 of the families, linkage to SPG7 locus was excluded. With one of the markers chosen for SPG7 restriction analysis was not successful, giving rise to inconclusive results in the other five families. Therefore, either new markers should have been chosen for this locus or exons of the paraplegin gene should be analyzed by DNA sequencing. Direct sequencing of seventeen exons of this gene for families H36, H46, H50, H52, and H55 is currently being performed.

Age of onset in the patients in these five families varies from 14 to 33 years old. Two families had pure HSP while other three were classified as complicated with phenotypes ranging from peripheral neuropathy to TCC and epileptic seizures. Since both pure and complicated types and even ARHSP-TCC are observed in SPG7-associated HSP (Stevanin

et al., 2006), exclusion of the locus is not possible by only evaluating the clinical phenotype.

6.1.3. Haplotype Analysis of SPG11 Locus

SPG11 is the most common ARHSP type and is associated with ARHSP with thin corpus callosum. Additional symptoms observed include urinary incontinence, late distal amyotrophy, cerebellar ataxia and mental deterioration (Lossos et al., 2006).

In family H45, an SPG11 haplotype segregating with the disease was observed. Two different softwares calculated the LOD score as 1.1767, a value below 3.0, which is the threshold for accepting linkage, with a 5% chance of error. However, the possible maximum two-point LOD score was calculated as 2.05 for this family. Although the critical threshold cannot be reached, when family size and DNA samples available are considered, it may be possible to assume this LOD score as significant. However, analysis on other family members can be performed to obtain higher LOD score values. Family H45 presented a complicated phenotype with peripheral neuropathy, dementia and mental retardation. Although TCC is not observed, the family is still a strong candidate for linkage to SPG11 locus.

For the same locus, patients P463 and P627 were found to be homozygous with the two markers analyzed. However, DNA samples were not available from unaffected individuals and linkage could not be tested further. The clinical phenotype of patients in P627 family was not reported but the symptoms including, distal weakness, TCC, cortical atrophy, pes cavus and mental impairment, observed in patients of family P463 correlated with the SPG11-associated HSP symptoms indicated in the literature. The clinician referring this family had also suggested the SPG11 as the causative gene.

For families H36 and H50, both affected and unaffected individuals were found to be homozygous for the markers of SPG11 locus, giving rise to non-informative results in these families. Direct sequencing can be performed but sequencing of 40 exons of the spatacsin is not a time and cost-efficient way. Therefore, haplotype analysis can be repeated by other SNP markers to test linkage first and then, if linkage is observed, exons

can be sequenced to identify causative mutation. As for their cilinical phenotypes, both of the families have complicated HSP. H36 has peripheral neuropathy, and H50 is classified as ARHSP-TCC, consistently with the SPG11-associated HSP.

Linkage to SPG11 locus was excluded in other ten families. Frequency of SPG11-associated HSP is 21% in all ARHSP cases and 59% in ARHSP-TCC type. One family in a total of 15-family-cohort and in a total of ARHSP-TCC group with four families is lower than the general frequency of SPG11-related HSP, in the literature. However, if another family among the families that gave non-informative or inconclusive was linked to SPG11, the frequency would be near to the expected value.

6.1.4. Haplotype Analysis of SPG15 Locus

SPG15 is the second most common type of ARHSP (Boukhris et al., 2008). Early disease onset with severe progression is observed. Additional symptoms in SPG15-associated HSP include TCC, mental retardation, maculopathy, distal amyotrophy, and mild cerebellar signs.

Family H29 was found to have a SPG15 haplotype segregating with the disease for two markers defining the SPG15 interval. Both affected and unaffected individuals analyzed were found to be homozygous for the other SNP marker (rs1955468). LOD score was calculated as 1.010, which is below critical value 3.0. The possible maximum two-point LOD score was calculated as 1.204 for this family. However, since FastSLink software used to calculate this maximum LOD score cannot perform analysis when there is o loop in the pedigree such as in H29 family. Thus, the maximum LOD score was calculated after breaking the loop so that the calculated value may not represent the real maximum LOD value of this family. However, the maximum LOD score of the family is assumed around 1.204. Critical threshold cannot be reached for this family, however, when the possible maximum LOD score and haplotype segregating with the disease are considered, linkage to SPG15 locus can be assumed for H29 family. Furthermore, clinical phenotype was associated with TCC, sphincter muscle disturbances, cerebellar atrophy, decreased vibration, pigmentation abnormality and cerebellar signs; and the observed early disease onset make the family H29 a strong candidate for SPG15-associated HSP.

The markers analyzed for SPG15 locus gave non-informative results for family P627 because the patients were found to be homozygous for these markers. To prove linkage other markers can be chosen and then, direct sequencing can be performed. Direct sequencing without proving linkage may not be an effective method due to high number of exons (41 exons) of the spastizin gene.

In thirteen of the families, linkage to SPG15 locus was excluded. One family linked to SPG15 locus in our 15-family-cohort is in consistence with the 15% frequency of SPG15-associated HSP among ARHSP cases.

6.1.5. Haplotype Analysis of SPG20 Locus

SPG20 is associated with complicated HSP, also known as Troyer syndrome (TRS). A founder mutation (1110delA) has been identified in the spartin gene in Old Order Amish families that causes TRS. In a recent study, a novel deletion mutation (c.364_365delAT) was reported in two Omani families.

In fourteen of the families, linkage to SPG20 locus was excluded. Affected individuals in family H6 were found to be homozygous for two of three markers analyzed for SPG20 locus. Since DNA samples from unaffected individuals were not available, the markers gave inconclusive results. Another SNP marker can be analyzed or direct sequencing of the eight exons of the spartin gene can also be performed to conclude this part of the study. Mutation observed in Omani families shows that SPG20-associated HSP is not solely restricted to Amish families. However, our family presents a pure phenotype thus; linkage to SPG20 locus would be highly unlikely.

6.1.6. Haplotype Analysis of SPG21 Locus

SPG21 is associated with an adult onset ARHSP-TCC with dementia, which is also known as Mast syndrome. A founder mutation in Old Order Amish families gives rise to disease. In the literature, further mutations have not been identified in another population. Therefore, linkage to SPG21 locus was not expected in our cohort.

Although linkage to SPG21 locus was excluded in fourteen of the families, a conclusive result could not be obtained for family H38. All of the individuals analyzed in the family were homozygous for the two markers on SPG21 locus. Family H38 had ARHSP-TCC with adult onset (>40 years), a phenotype that resembles to that of SPG21-associated HSP but without dementia. This observation brings the necessity for exclusion of the locus in the family. For this purpose, other markers can be chosen for linkage analysis or presence of the founder mutation can be tested in further studies.

Common haplotypes may be observed among different human populations with variable frequency. The SNP markers chosen from HapMap database were highly heterozygous for CEU: CEPH (Utah residents with ancestry from northern and western Europe) population. However, in this study, both affected and unaffected individuals analyzed in four of the families were found to be homozygous for the markers in at least one of the tested loci. These non-informative results indicate the importance of a SNP database study that will include the Turkish population.

In this study, linkage to all tested loci was excluded in four families. Though the possibility of linkage to other known loci is not excluded in our families, there are few cases (one family or two families) reported for those loci. Therefore, it can be concluded that there is further heterogeneity for ARHSP.

7. CONCLUSION

This study is important for understanding the genetic basis of autosomal recessive HSP in Turkish families. Haplotype analyses in fifteen families revealed a linkage to SPG11 locus in one and to SPG15 locus in one other family. In three families, linkage to all tested loci was excluded. Results were non-informative for four families for one of tested loci. In two families, patients were homozygous for the markers for at least one locus. However, linkage is not proved for these families because DNA samples of the unaffected individuals were not available. In six families, the base on the SNP marker site could not be determined for at least one of the markers, giving inconclusive results. In two of the families, restriction analyses gave both non-informative and inconclusive results. To obtain informative and conclusive results, other SNP markers should be chosen and linkage to related loci should be tested with these new markers. The families, for which linkage to tested loci was excluded, can be analyzed in a study to associate the disease with other ARHSP loci.

APPENDIX A: FAMILIES EXCLUDED FOR SPG5 LOCUS

Haplotypes of the families excluded for SPG5 locus by using the SNP markers spanning the SPG5 locus are given in Figure A.1 through Figure A.13.

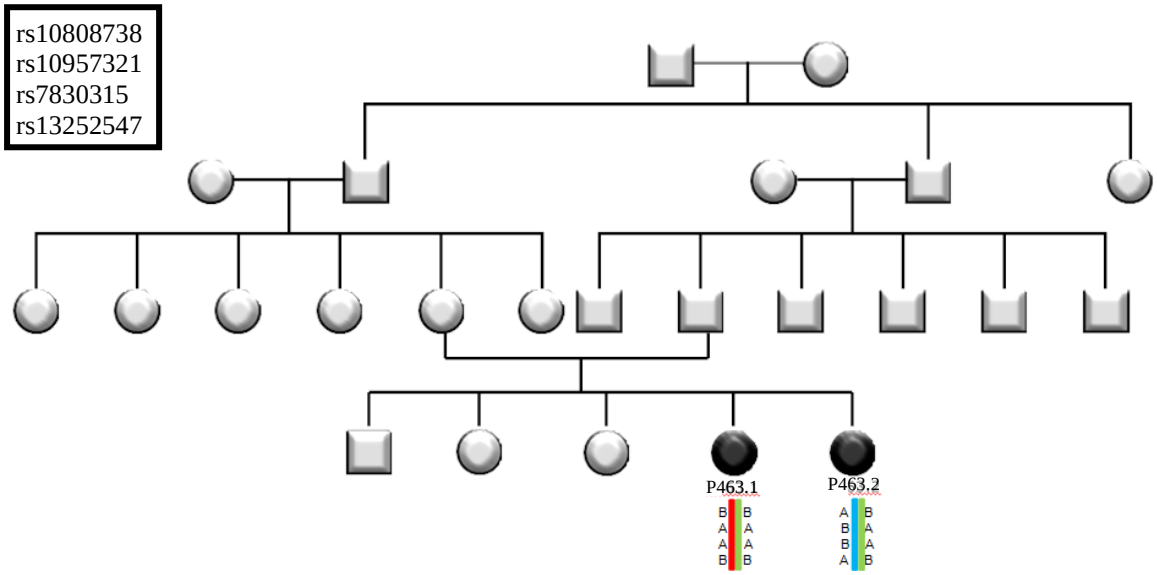


Figure A.1. Haplotype of the family P463 for the markers spanning the SPG5 locus. The box on the left shows the order of the SNP markers used for this locus.

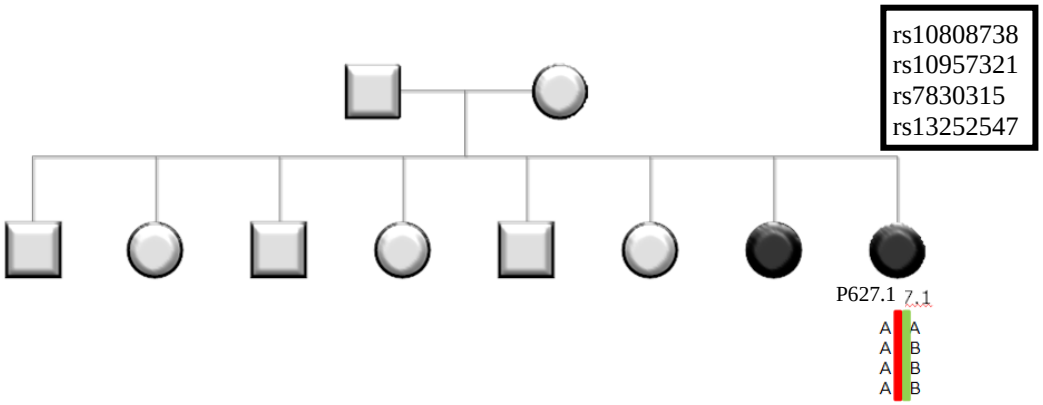


Figure A.2. Haplotype of the family P627 for the markers spanning the SPG5 locus. The box on the right shows the order of the SNP markers used for this locus.

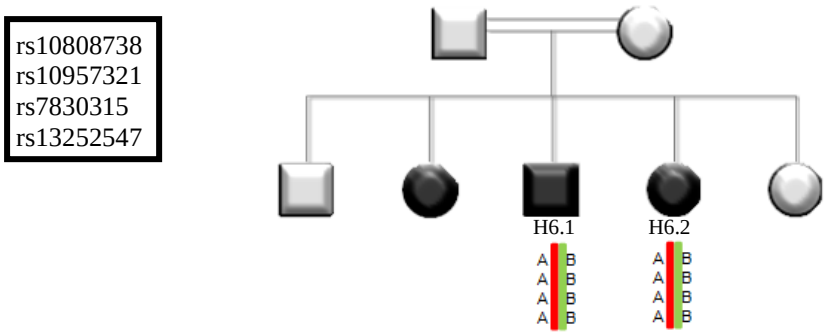


Figure A.3. Haplotype of the family H6 for the markers spanning the SPG5 locus. The box on the left shows the order of the SNP markers used for this locus.

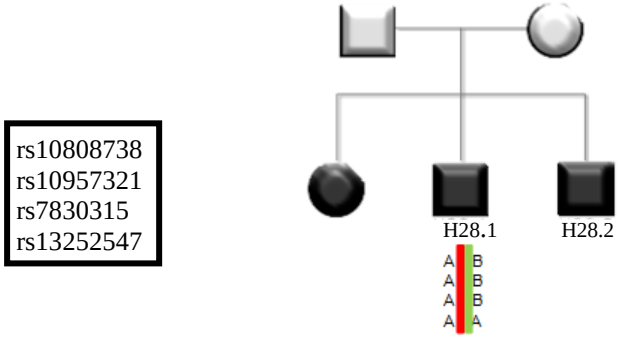


Figure A.4. Haplotype of the family H28 for the markers spanning the SPG5 locus. The box on the left shows the order of the SNP markers used for this locus.

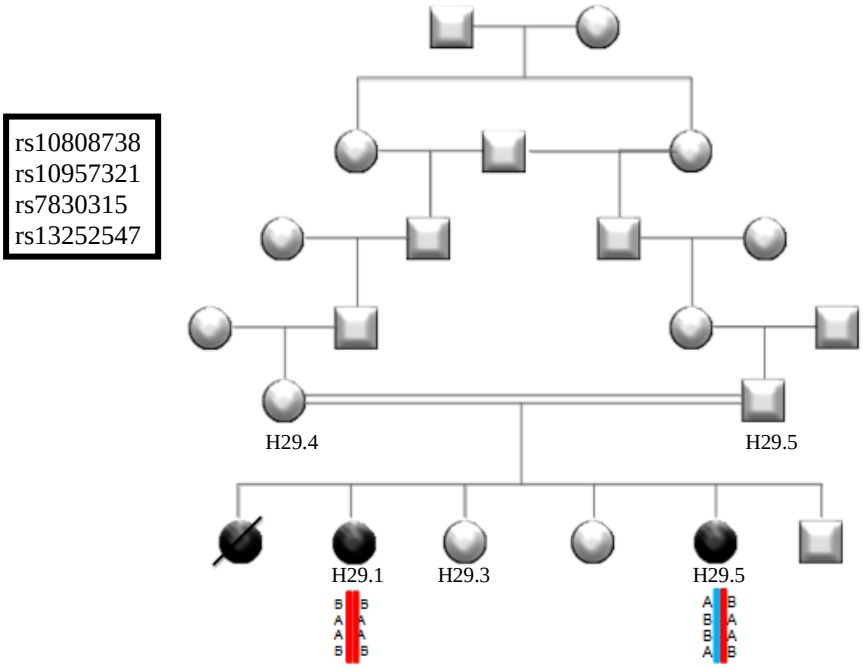


Figure A.5. Haplotype of the family H29 for the markers spanning the SPG5 locus. The box on the left shows the order of the SNP markers used for this locus.

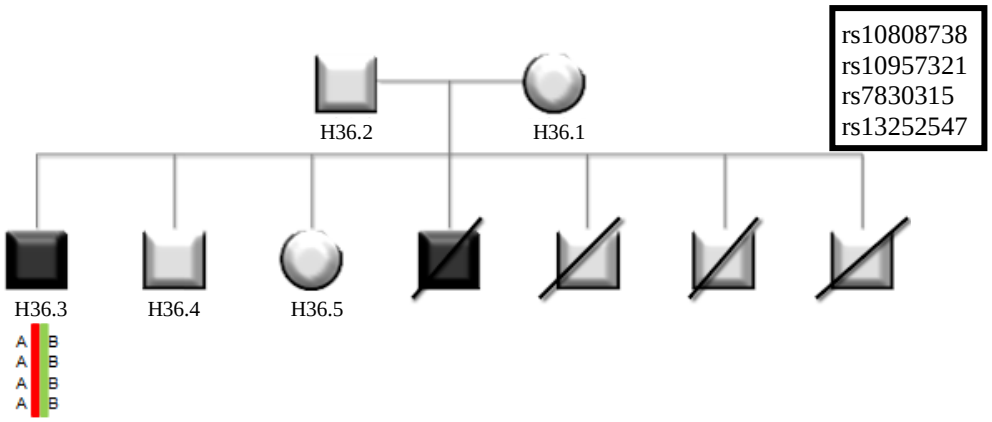


Figure A.6. Haplotype of the family H36 for the markers spanning the SPG5 locus. The box on the right shows the order of the SNP markers used for this locus.

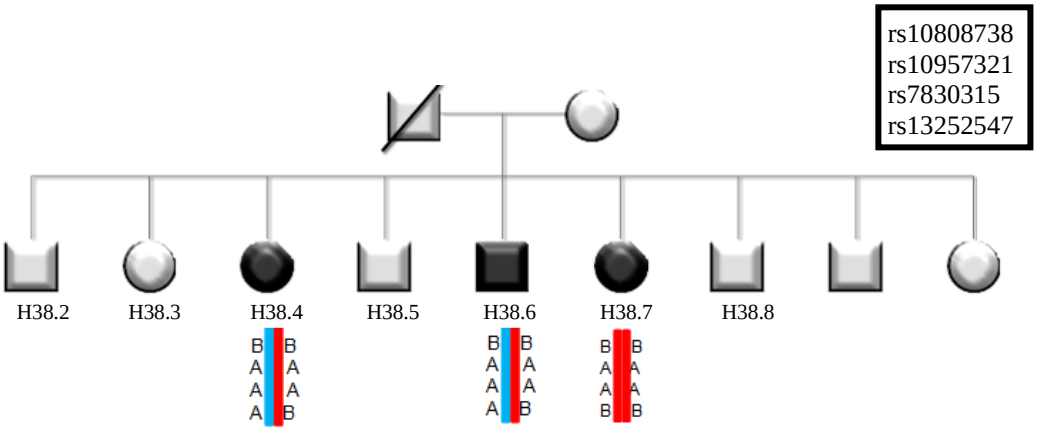


Figure A.7. Haplotype of the family H38 for the markers spanning the SPG5 locus. The box on the right shows the order of the SNP markers used for this locus.

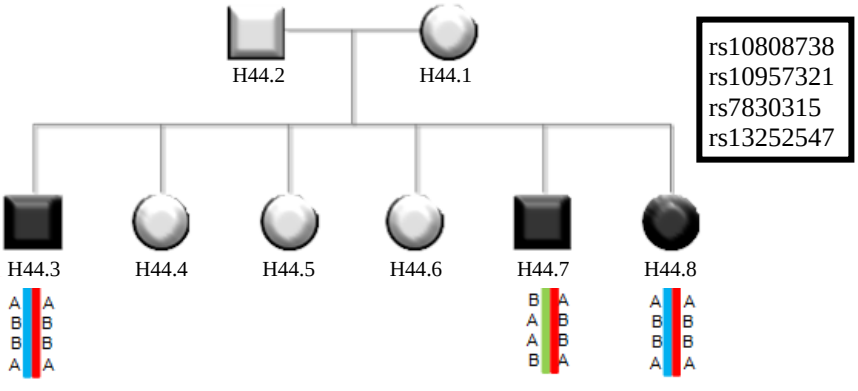


Figure A.8. Haplotype of the family H44 for the markers spanning the SPG5 locus. The box on the right shows the order of the SNP markers used for this locus.

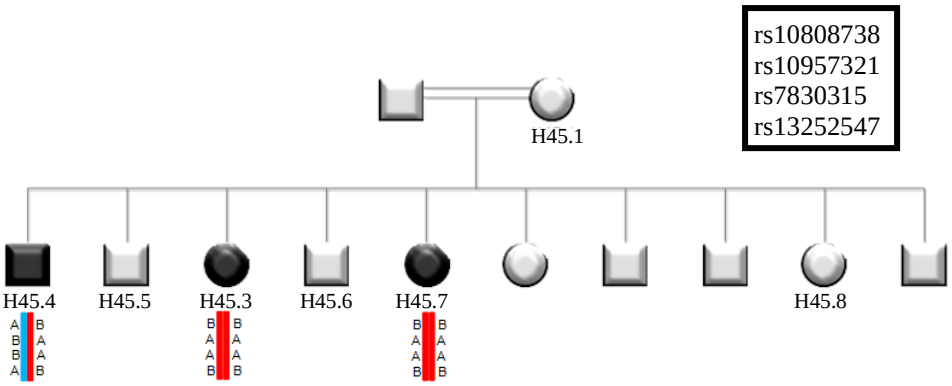


Figure A.9. Haplotype of the family H45 for the markers spanning the SPG5 locus. The box on the right shows the order of the SNP markers used for this locus.

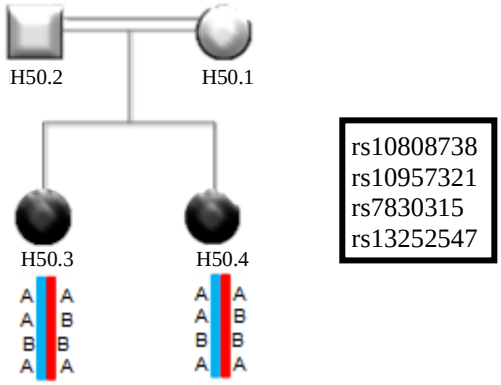


Figure A.10. Haplotype of the family H50 for the markers spanning the SPG5 locus. The box on the right shows the order of the SNP markers used for this locus.

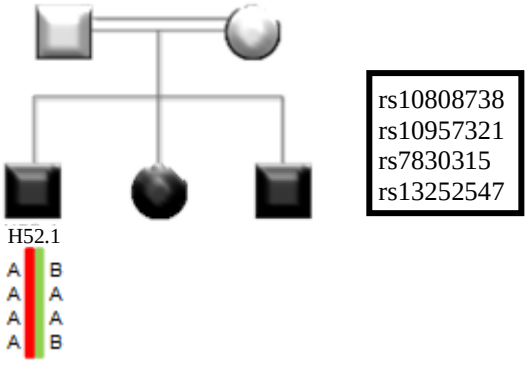


Figure A.11. Haplotype of the family H52 for the markers spanning the SPG5 locus. The box on the right shows the order of the SNP markers used for this locus.

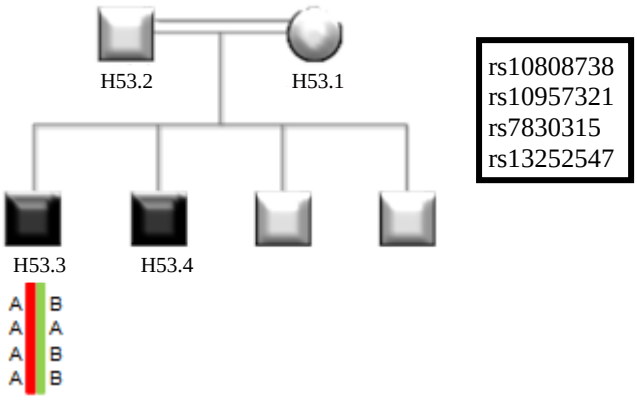


Figure A.12. Haplotype of the family H53 for the markers spanning the SPG5 locus. The box on the right shows the order of the SNP markers used for this locus.

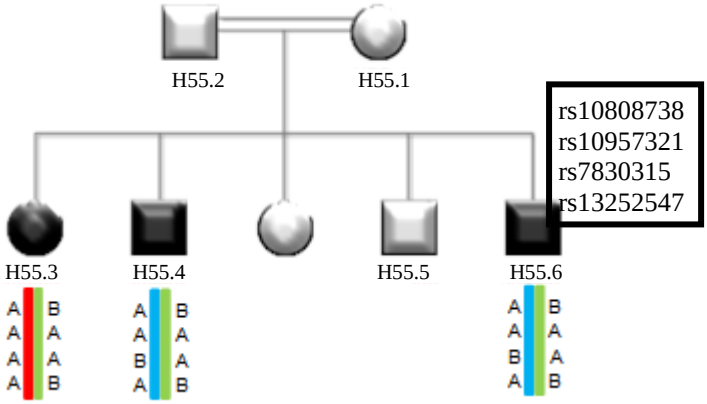


Figure A.13. Haplotype of the family H55 for the markers spanning the SPG5 locus. The box on the right shows the order of the SNP markers used for this locus.

APPENDIX B: FAMILIES EXCLUDED FOR SPG7 LOCUS

Haplotypes of the families excluded for SPG7 locus by using the SNP markers spanning the SPG7 locus are given in Figure B.1 through Figure B.9. Nature of the SNP marker rs8046182 could not be determined in any of the families due to improper functioning of the restriction enzyme.

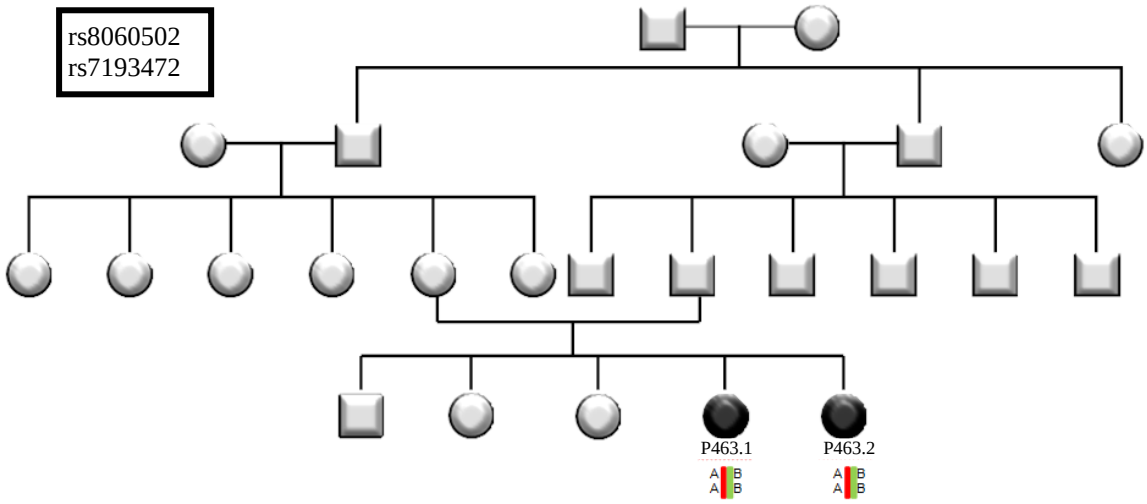


Figure B.1. Haplotype of the family P463 for the markers spanning the SPG7 locus. The box on the left shows the order of the SNP markers used for this locus.

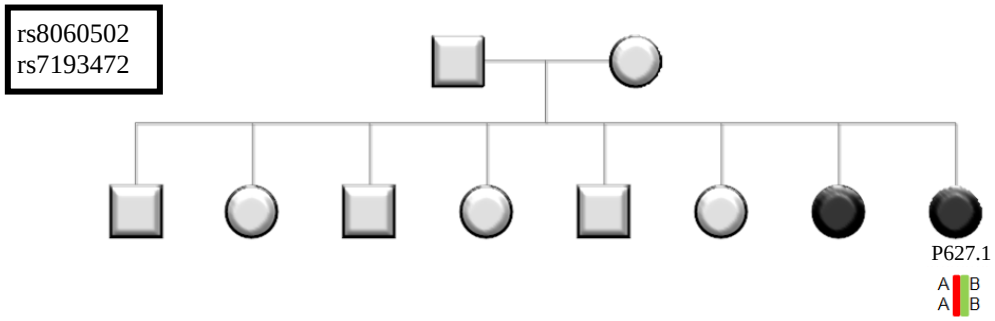


Figure B.2. Haplotype of the family P627 for the markers spanning the SPG7 locus. The box on the left shows the order of the SNP markers used for this locus.

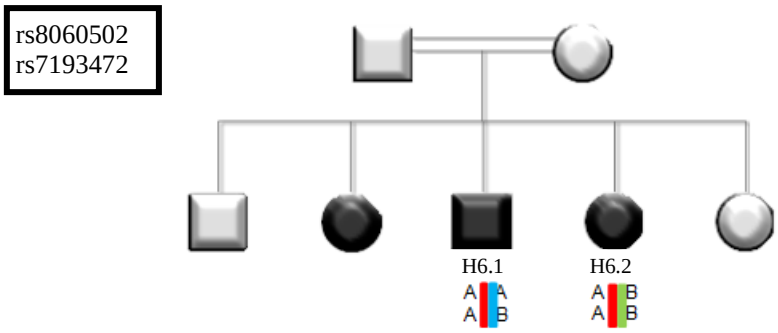


Figure B.3. Haplotype of the family H6 for the markers spanning the SPG7 locus. The box on the left shows the order of the SNP markers used for this locus.

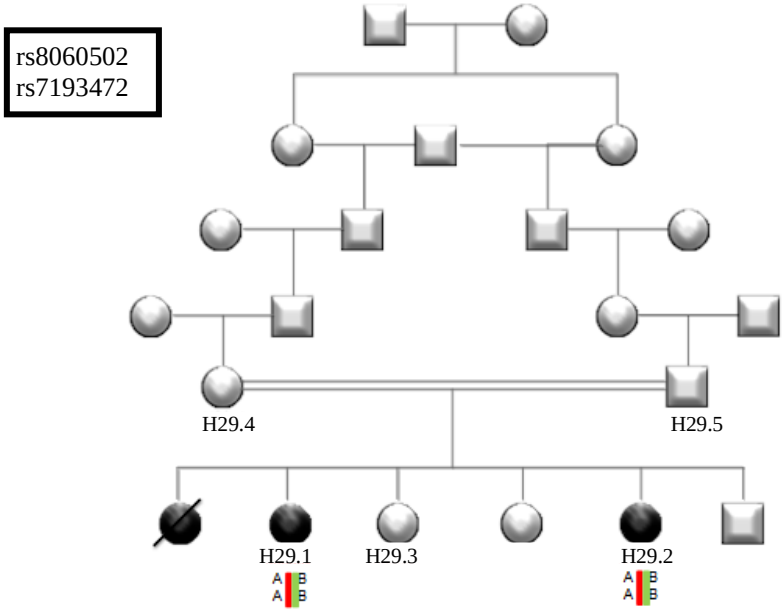


Figure B.4. Haplotype of the family H29 for the markers spanning the SPG7 locus. The box on the left shows the order of the SNP markers used for this locus.

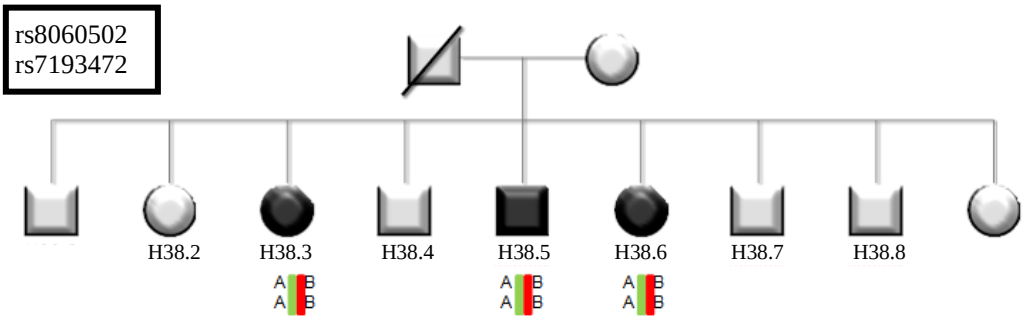


Figure B.5. Haplotype of the family H38 for the markers spanning the SPG7 locus. The box on the left shows the order of the SNP markers used for this locus.

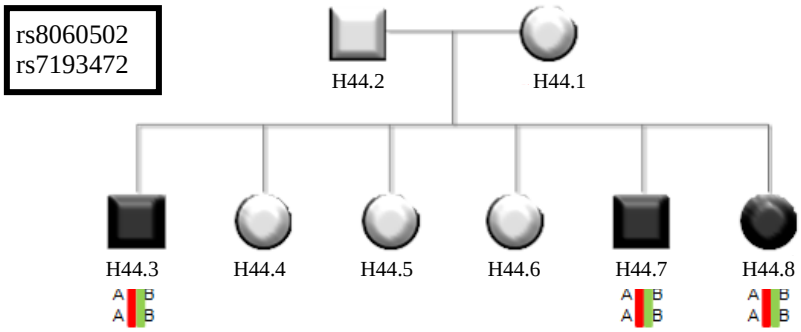


Figure B.6. Haplotype of the family H44 for the markers spanning the SPG7 locus. The box on the left shows the order of the SNP markers used for this locus.

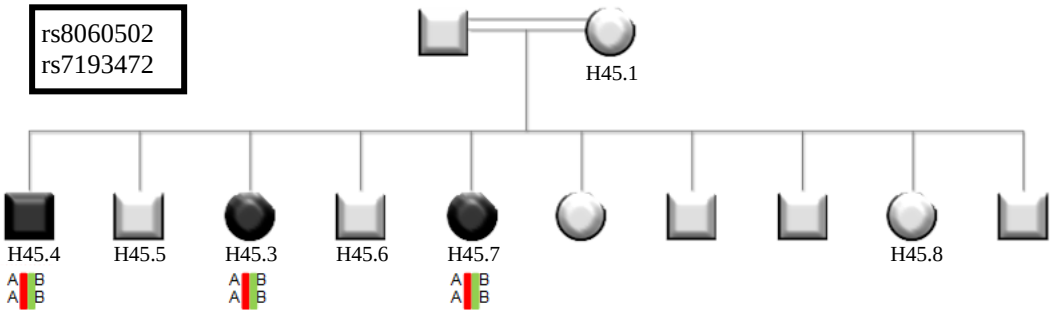


Figure B.8. Haplotype of the family H45 for the markers spanning the SPG7 locus. The box on the left shows the order of the SNP markers used for this locus.

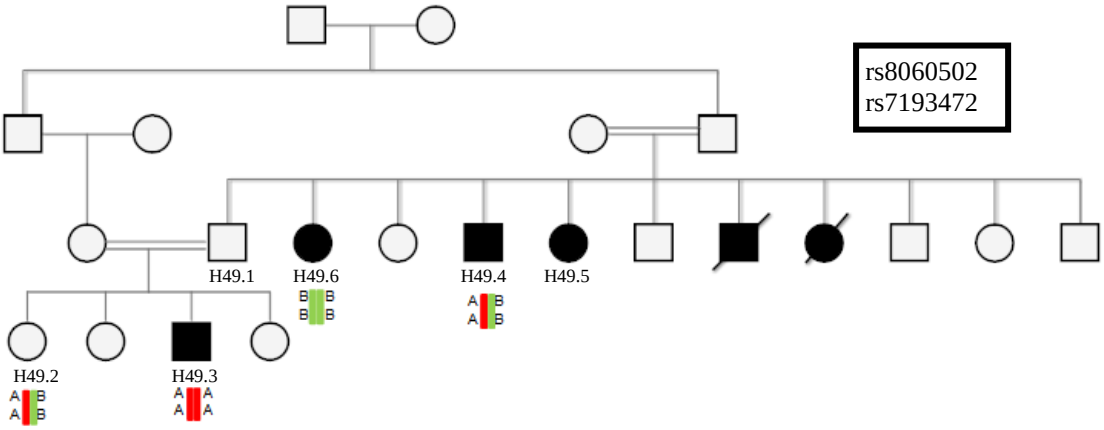


Figure B.9. Haplotype of the family H49 for the markers spanning the SPG7 locus. The box on the right shows the order of the SNP markers used for this locus.

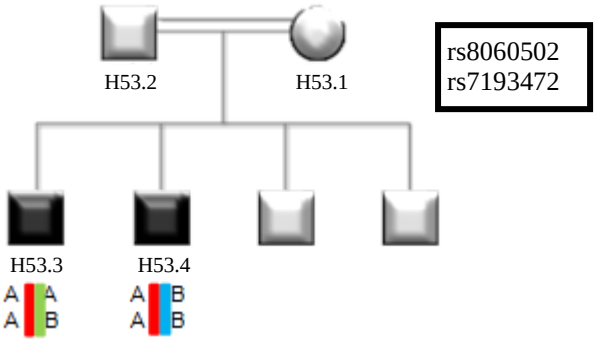


Figure B.9. Haplotype of the family H53 for the markers spanning the SPG7 locus. The box on the right shows the order of the SNP markers used for this locus.

APPENDIX C: FAMILIES EXCLUDED FOR SPG11 LOCUS

Haplotypes of the families excluded for SPG11 locus by using the SNP markers spanning the SPG11 locus are given in Figure C.1 through Figure C.10.

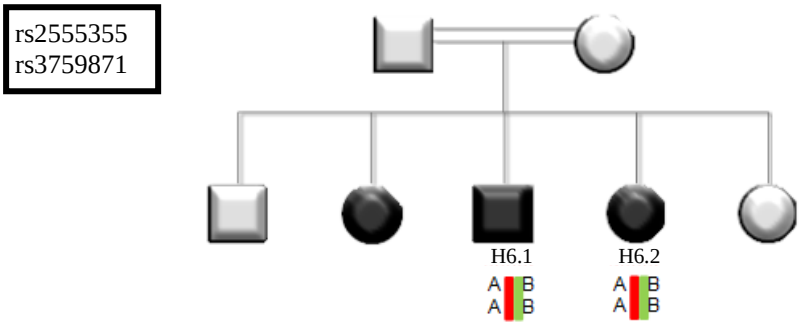


Figure C.1. Haplotype of the family H6 for the markers spanning the SPG11 locus. The box on the left shows the order of the SNP markers used for this locus.

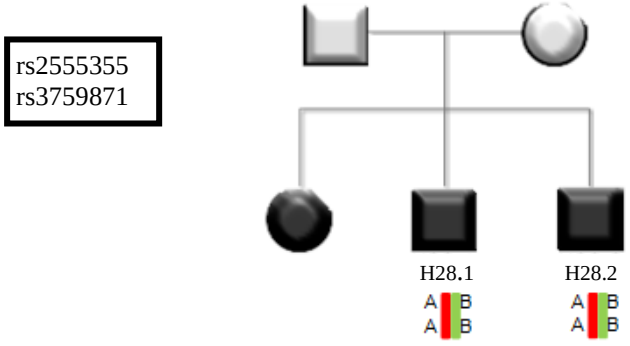


Figure C.2. Haplotype of the family H28 for the markers spanning the SPG11 locus. The box on the left shows the order of the SNP markers used for this locus.

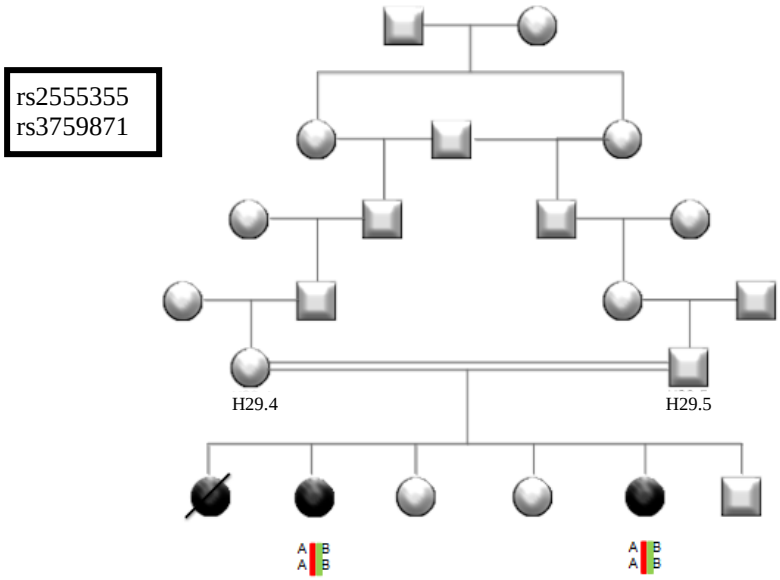


Figure C.3. Haplotype of the family H29 for the markers spanning the SPG11 locus. The box on the left shows the order of the SNP markers used for this locus.

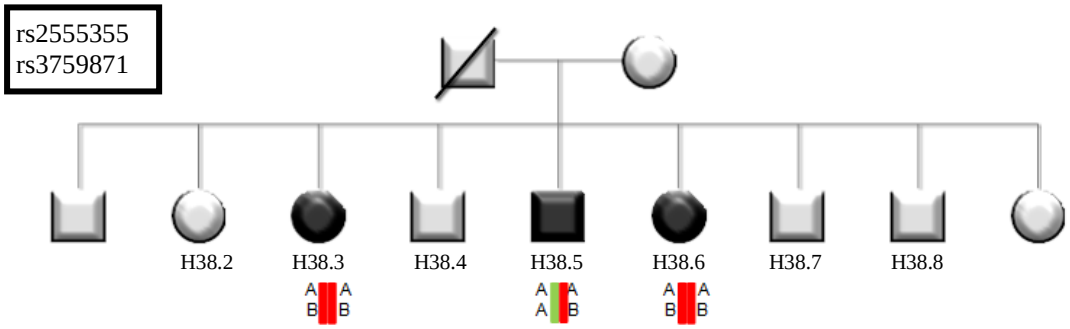


Figure C.4. Haplotype of the family H38 for the markers spanning the SPG11 locus. The box on the left shows the order of the SNP markers used for this locus.

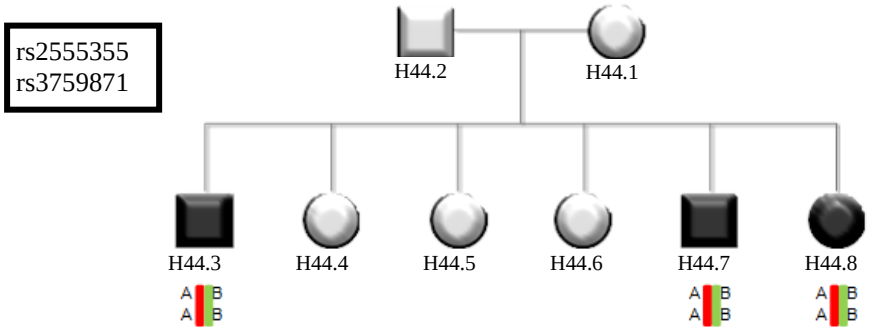


Figure C.5. Haplotype of the family H44 for the markers spanning the SPG11 locus. The box on the left shows the order of the SNP markers used for this locus.

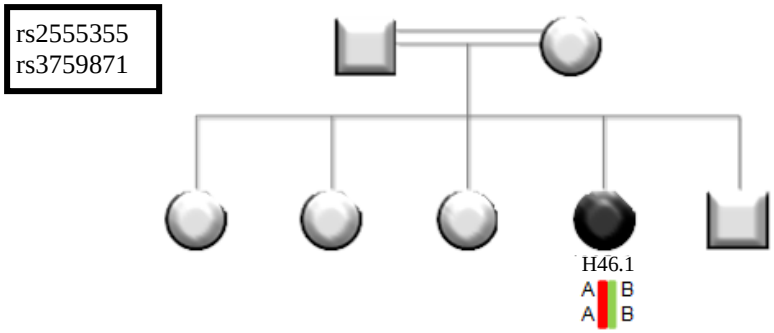


Figure C.6. Haplotype of the family H46 for the markers spanning the SPG11 locus. The box on the left shows the order of the SNP markers used for this locus.

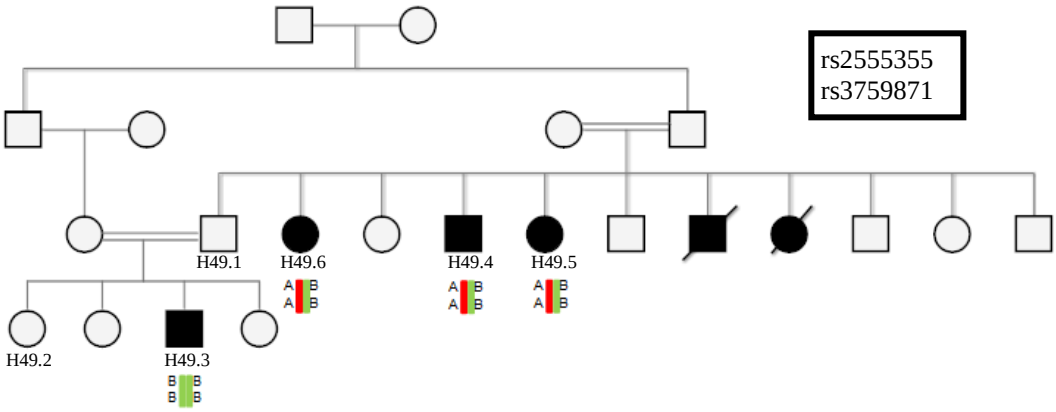


Figure C.7. Haplotype of the family H49 for the markers spanning the SPG11 locus. The box on the right shows the order of the SNP markers used for this locus.

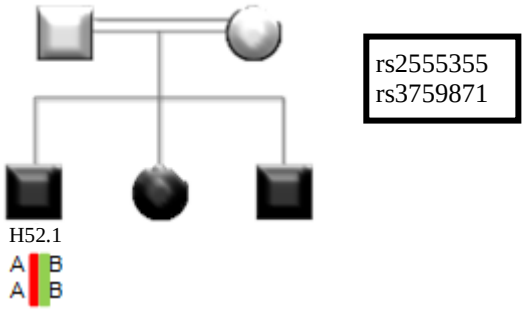


Figure C.8. Haplotype of the family H52 for the markers spanning the SPG11 locus. The box on the right shows the order of the SNP markers used for this locus.

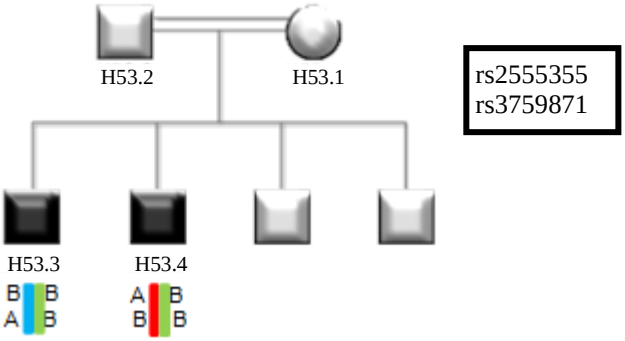


Figure C.9. Haplotype of the family H53 for the markers spanning the SPG11 locus. The box on the right shows the order of the SNP markers used for this locus.

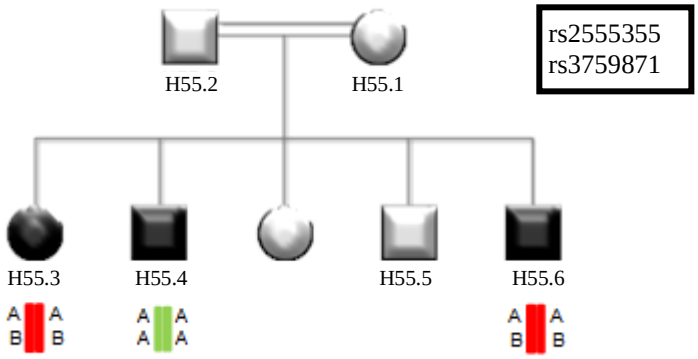


Figure C.10. Haplotype of the family H55 for the markers spanning the SPG11 locus. The box on the right shows the order of the SNP markers used for this locus.

APPENDIX D: FAMILIES EXCLUDED FOR SPG15 LOCUS

Haplotypes of the families excluded for SPG15 locus by using the SNP markers spanning the SPG11 locus are given in Figure D.1 through Figure D.12.

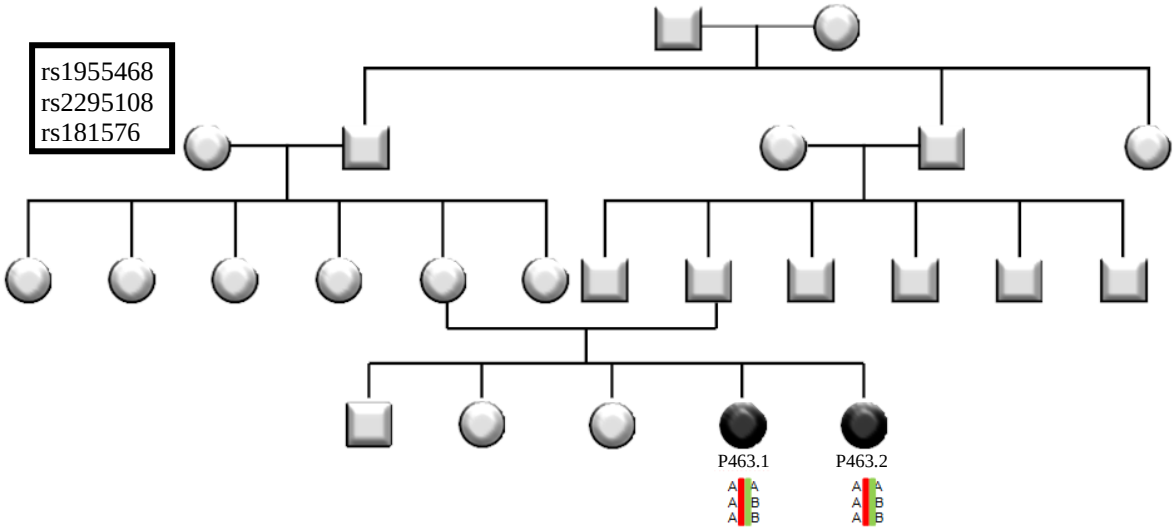


Figure D.1. Haplotype of the family P463 for the markers spanning the SPG15 locus. The box on the left shows the order of the SNP markers used for this locus.

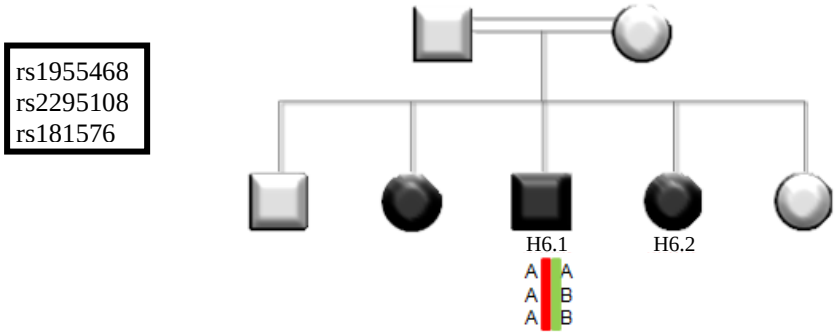


Figure D.2. Haplotype of the family H6 for the markers spanning the SPG15 locus. The box on the left shows the order of the SNP markers used for this locus.

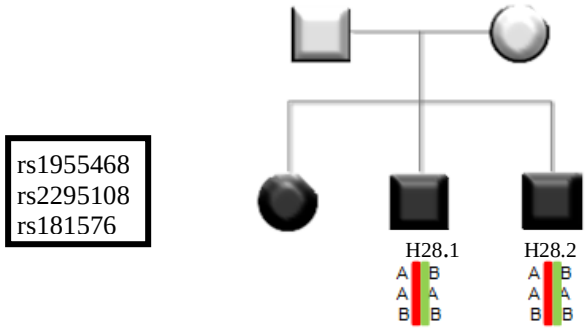


Figure D.3. Haplotype of the family H28 for the markers spanning the SPG15 locus. The box on the left shows the order of the SNP markers used for this locus.

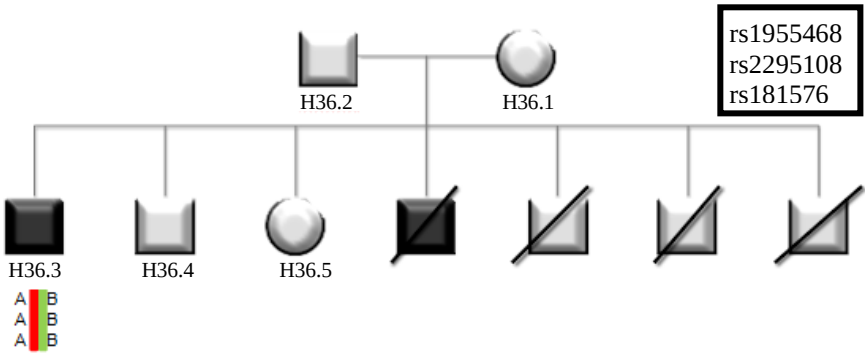


Figure D.4. Haplotype of the family H36 for the markers spanning the SPG15 locus. The box on the right shows the order of the SNP markers used for this locus.

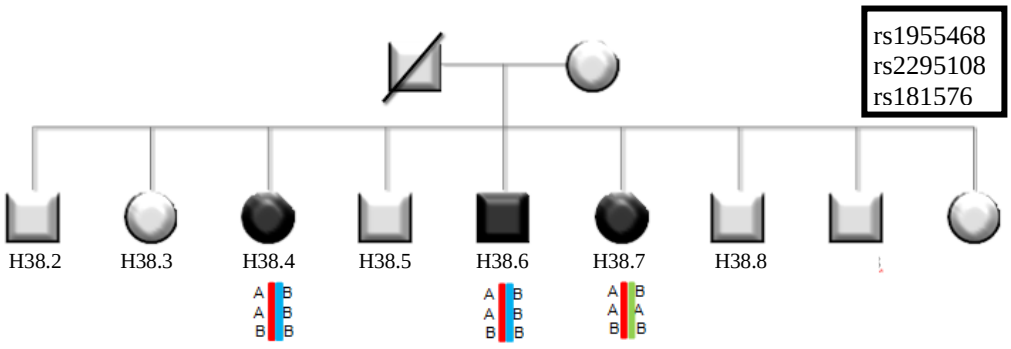


Figure D.5. Haplotype of the family H38 for the markers spanning the SPG15 locus. The box on the right shows the order of the SNP markers used for this locus.

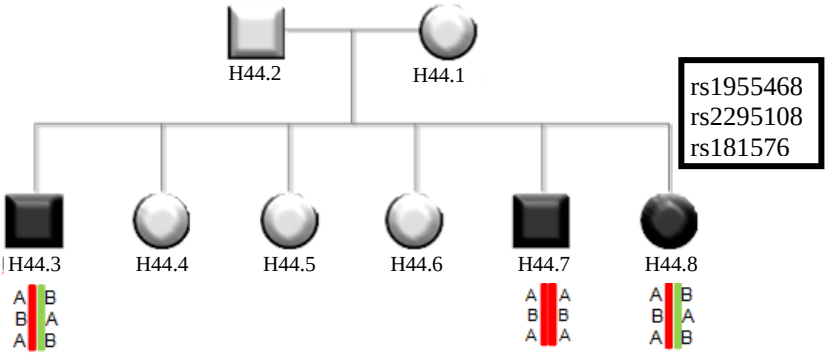


Figure D.6. Haplotype of the family H44 for the markers spanning the SPG15 locus. The box on the right shows the order of the SNP markers used for this locus.

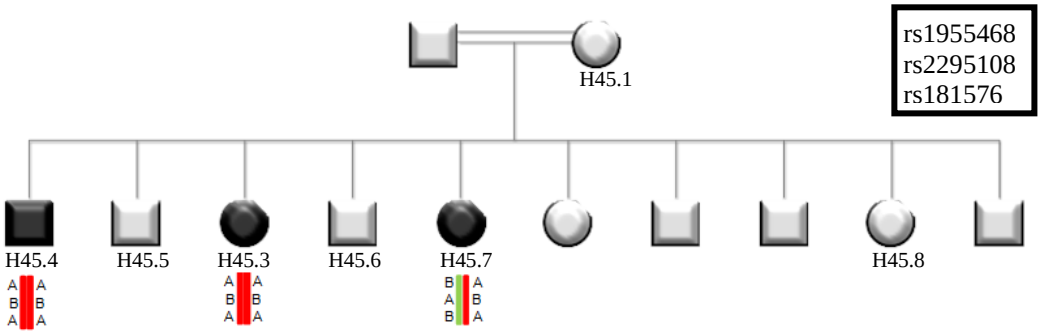


Figure D.7. Haplotype of the family H45 for the markers spanning the SPG15 locus. The box on the right shows the order of the SNP markers used for this locus.

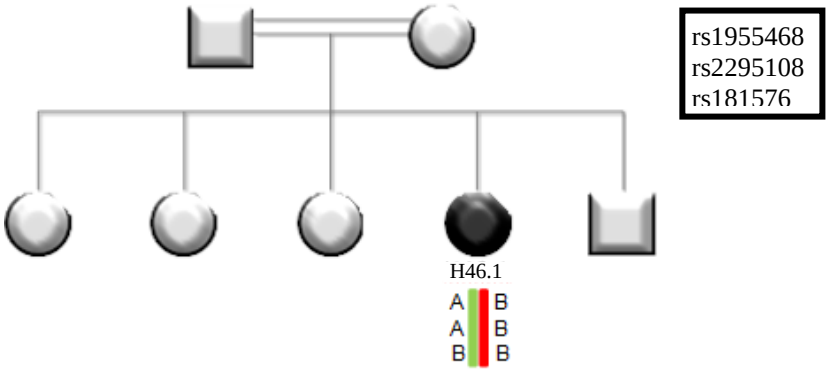


Figure D.8. Haplotype of the family H46 for the markers spanning the SPG15 locus. The box on the right shows the order of the SNP markers used for this locus.

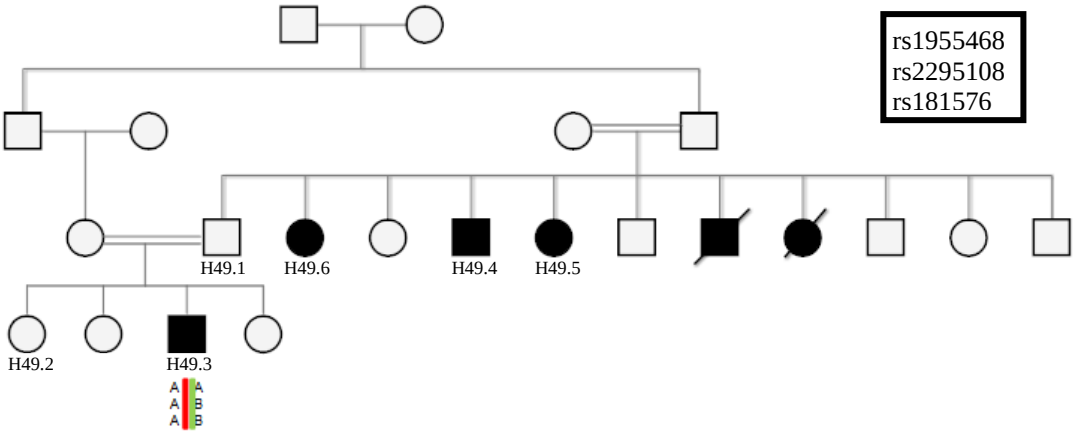


Figure D.9. Haplotype of the family H49 for the markers spanning the SPG15 locus. The box on the right shows the order of the SNP markers used for this locus.

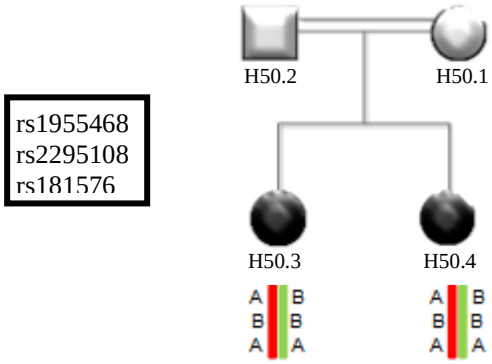


Figure D.10. Haplotype of the family H50 for the markers spanning the SPG15 locus. The box on the left shows the order of the SNP markers used for this locus.

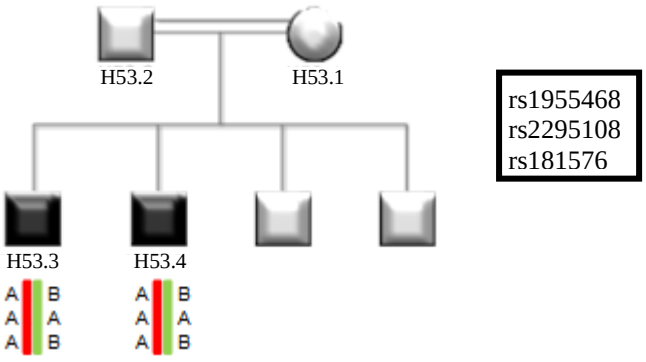


Figure D.11. Haplotype of the family H53 for the markers spanning the SPG15 locus. The box on the right shows the order of the SNP markers used for this locus

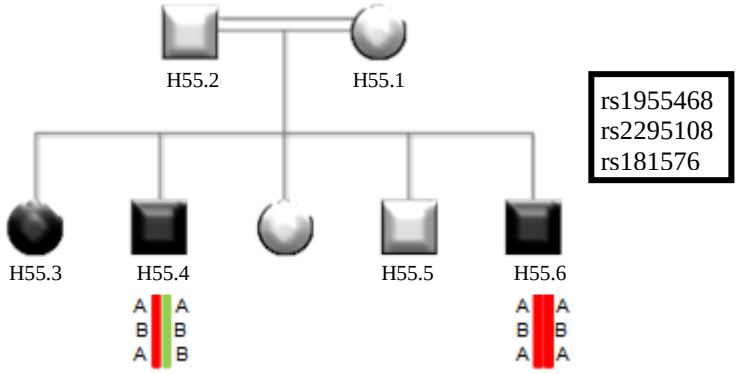


Figure D.12. Haplotype of the family H55 for the markers spanning the SPG15 locus. The box on the right shows the order of the SNP markers used for this locus.

APPENDIX E: FAMILIES EXCLUDED FOR SPG20 LOCUS

Haplotypes of the families excluded for SPG20 locus by using the SNP markers spanning the SPG11 locus are given in Figure E.1 through Figure E.13. Nature of the SNP marker rs7335635 could not be determined in any of the individuals due to improper functioning of the restriction enzyme.

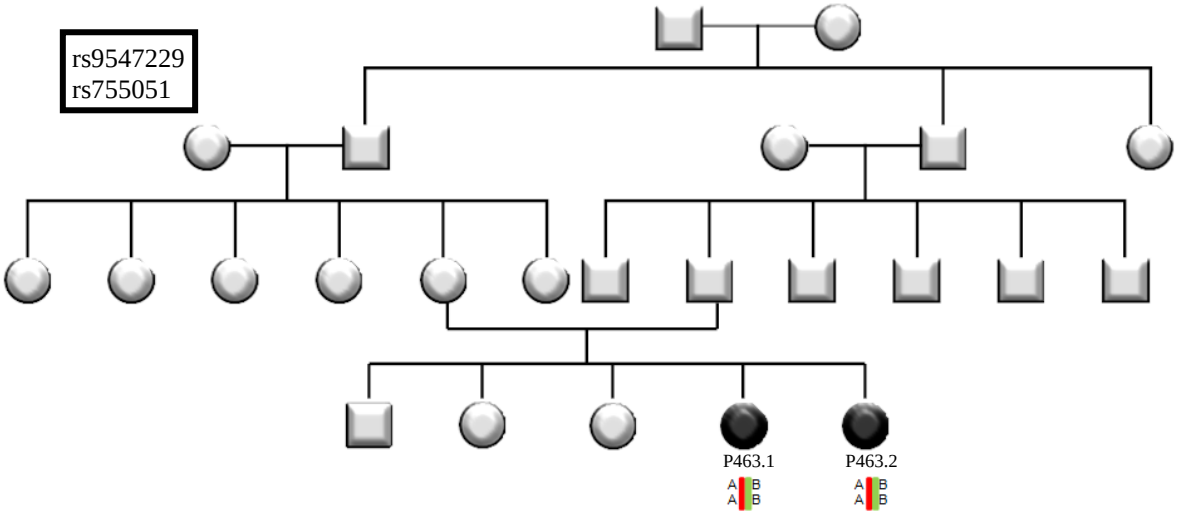


Figure E.1. Haplotype of the family P463 for the markers spanning the SPG20 locus. The box on the left shows the order of the SNP markers used for this locus.

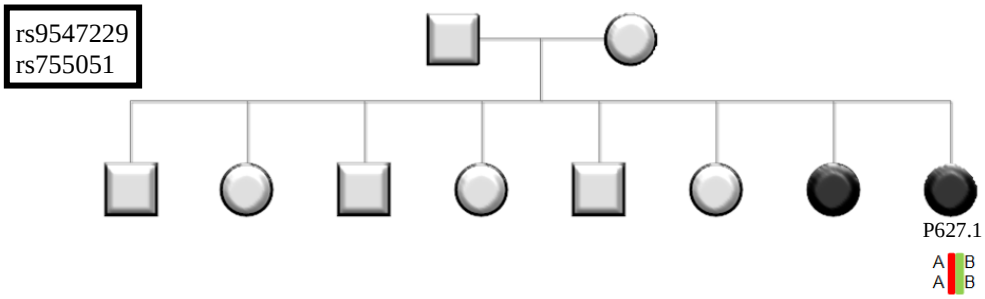


Figure E.2. Haplotype of the family P627 for the markers spanning the SPG20 locus. The box on the left shows the order of the SNP markers used for this locus.

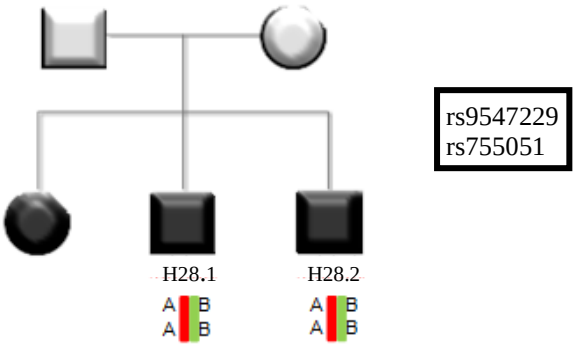


Figure E.3. Haplotype of the family H28 for the markers spanning the SPG20 locus. The box on the right shows the order of the SNP markers used for this locus.

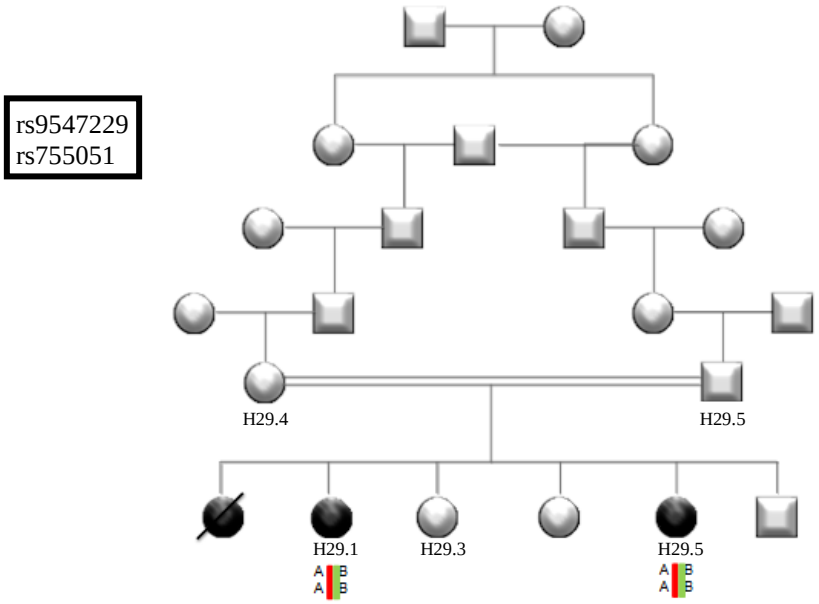


Figure E.4. Haplotype of the family H29 for the markers spanning the SPG20 locus. The box on the left shows the order of the SNP markers used for this locus.

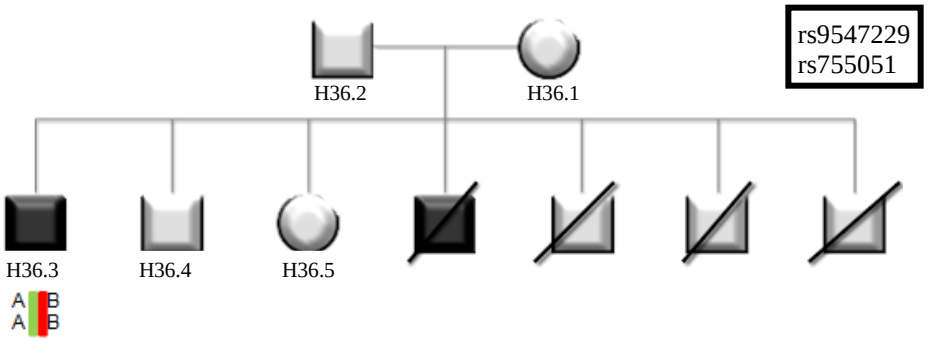


Figure E.5. Haplotype of the family H36 for the markers spanning the SPG20 locus. The box on the right shows the order of the SNP markers used for this locus.

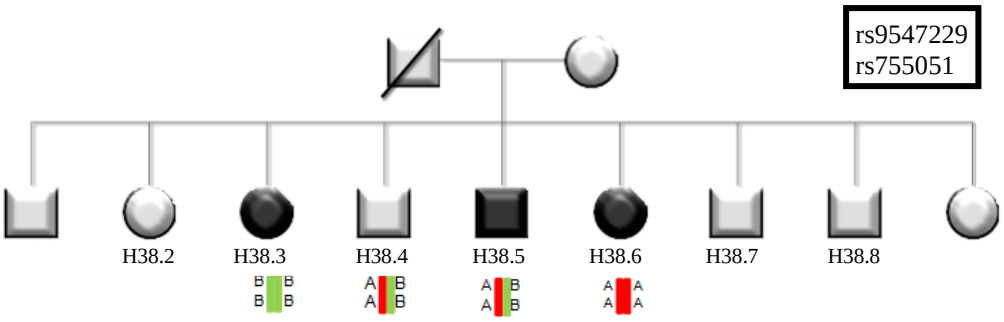


Figure E.6. Haplotype of the family H38 for the markers spanning the SPG20 locus. The box on the right shows the order of the SNP markers used for this locus.

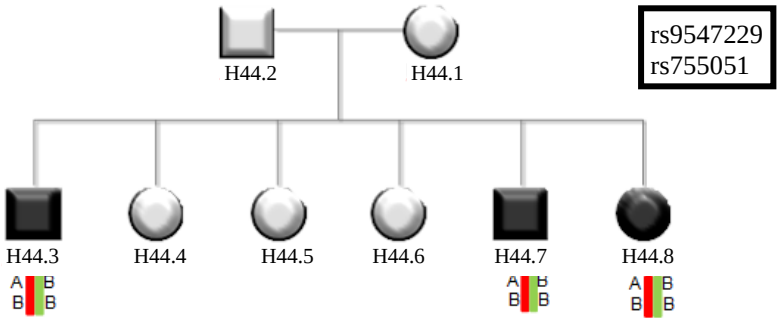


Figure E.7. Haplotype of the family H44 for the markers spanning the SPG20 locus. The box on the right shows the order of the SNP markers used for this locus.

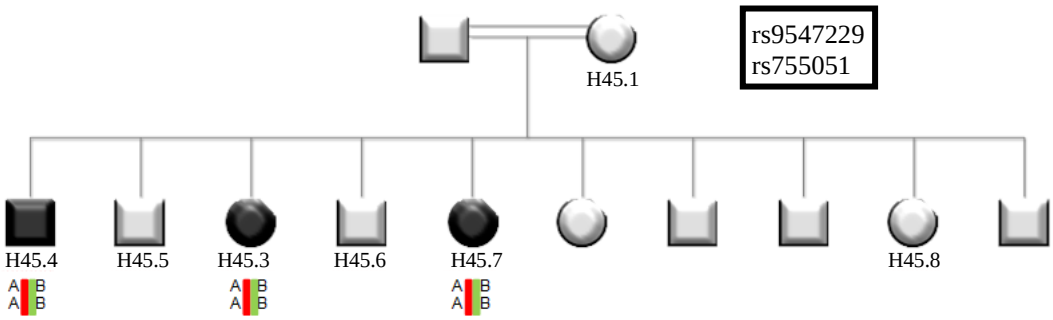


Figure E.8. Haplotype of the family H45 for the markers spanning the SPG20 locus. The box on the right shows the order of the SNP markers used for this locus.

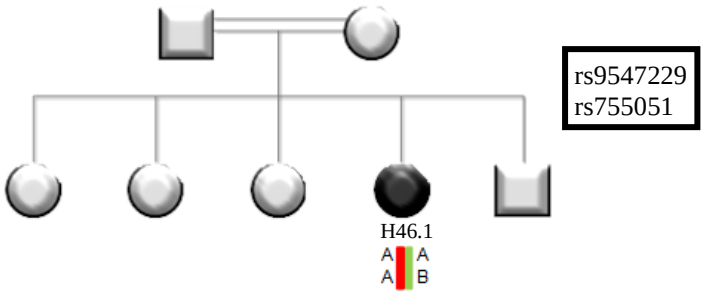


Figure E.9. Haplotype of the family H46 for the markers spanning the SPG20 locus. The box on the right shows the order of the SNP markers used for this locus.

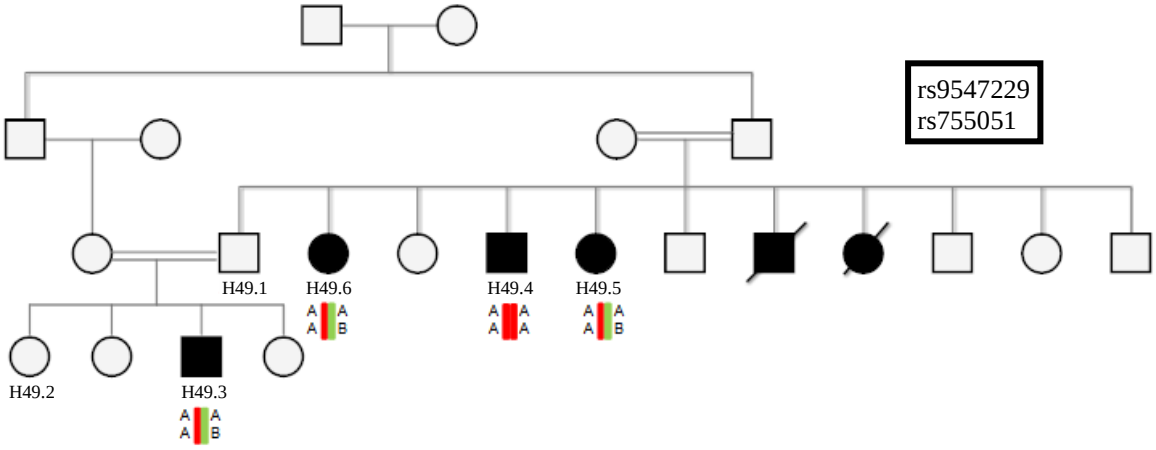


Figure E.10. Haplotype of the family H49 for the markers spanning the SPG20 locus. The box on the right shows the order of the SNP markers used for this locus.

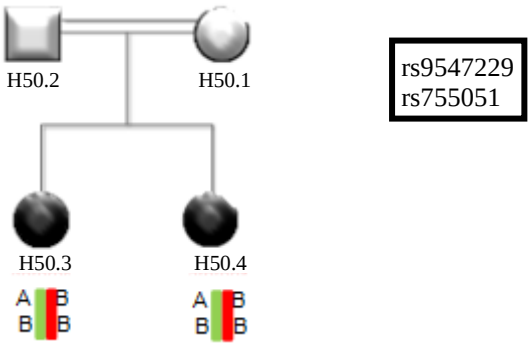


Figure E.11. Haplotype of the family H50 for the markers spanning the SPG20 locus. The box on the left shows the order of the SNP markers used for this locus.

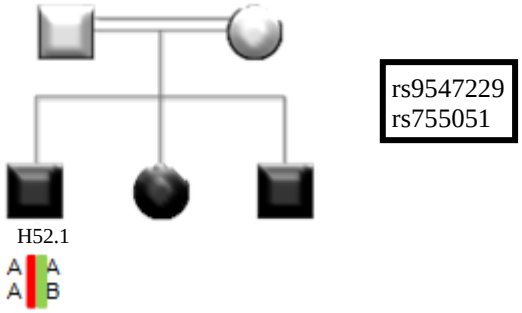


Figure E.12. Haplotype of the family H52 for the markers spanning the SPG20 locus. The box on the right shows the order of the SNP markers used for this locus.

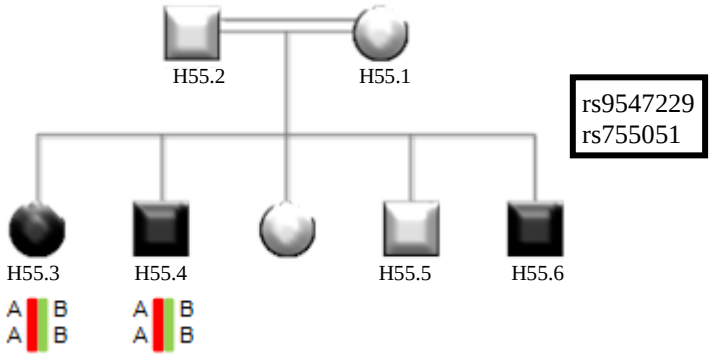


Figure E.13. Haplotype of the family H55 for the markers spanning the SPG20 locus. The box on the right shows the order of the SNP markers used for this locus.

APPENDIX F: FAMILIES EXCLUDED FOR SPG21 LOCUS

Haplotypes of the families excluded for SPG21 locus by using the SNP markers spanning the SPG21 locus are given in Figure F.1 through Figure F.13.

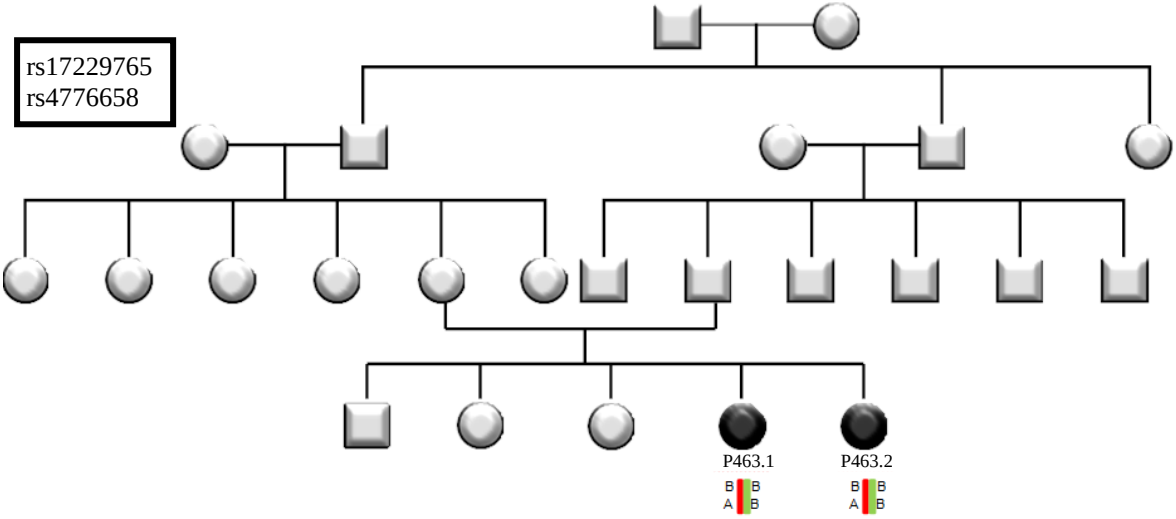


Figure F.1. Haplotype of the family P463 for the markers spanning the SPG21 locus. The box on the left shows the order of the SNP markers used for this locus.

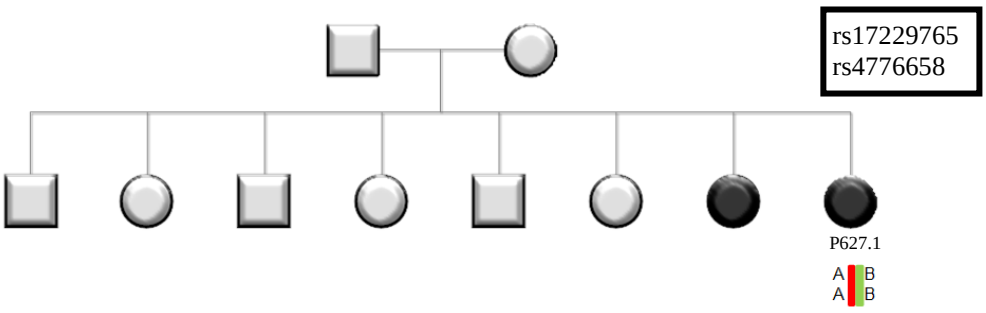


Figure F.2. Haplotype of the family P627 for the markers spanning the SPG21 locus. The box on the right shows the order of the SNP markers used for this locus.

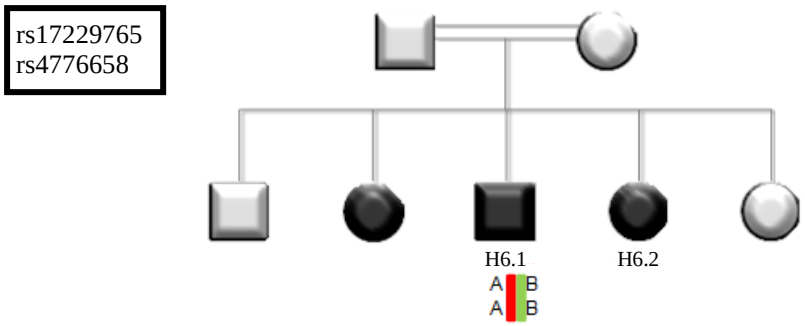


Figure F.3. Haplotype of the family H6 for the markers spanning the SPG21 locus. The box on the left shows the order of the SNP markers used for this locus.

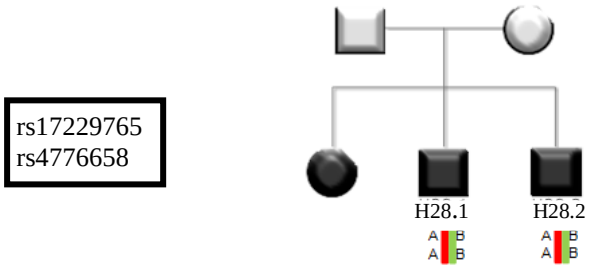


Figure F.4. Haplotype of the family H28 for the markers spanning the SPG21 locus. The box on the left shows the order of the SNP markers used for this locus.

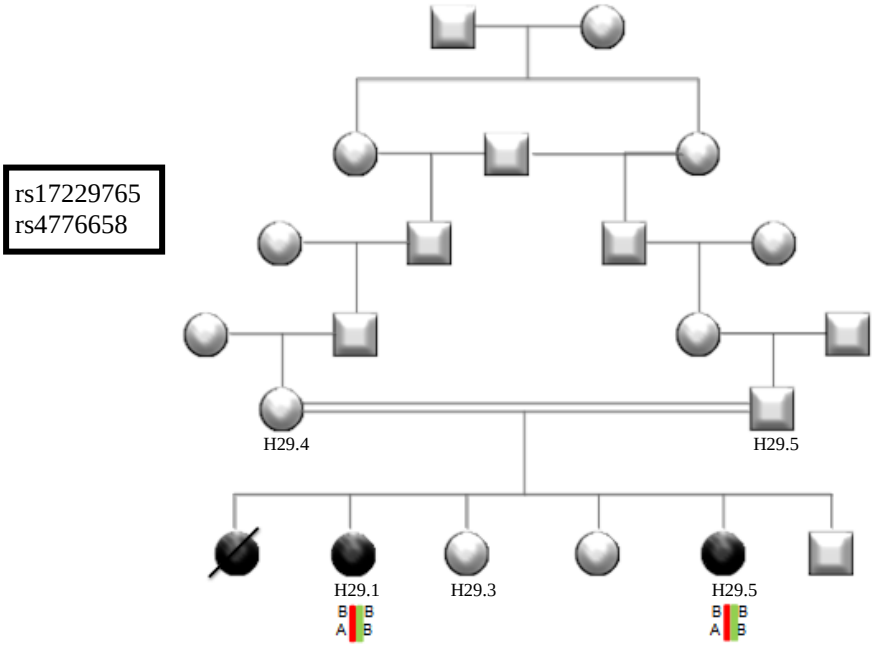


Figure F.5. Haplotype of the family H29 for the markers spanning the SPG21 locus. The box on the left shows the order of the SNP markers used for this locus.

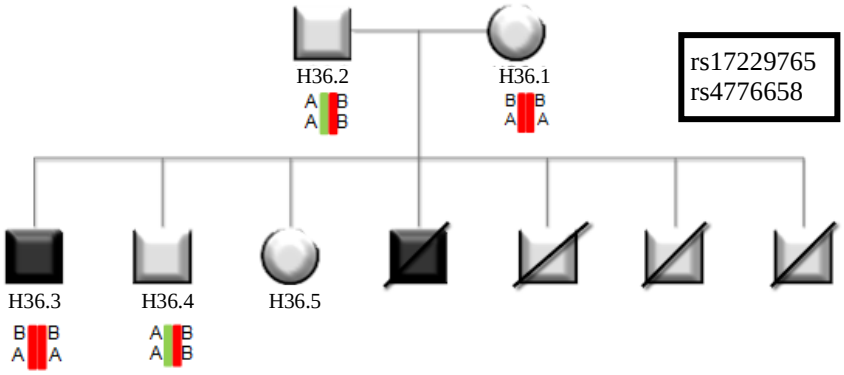


Figure F.6. Haplotype of the family H36 for the markers spanning the SPG21 locus. The box on the right shows the order of the SNP markers used for this locus.

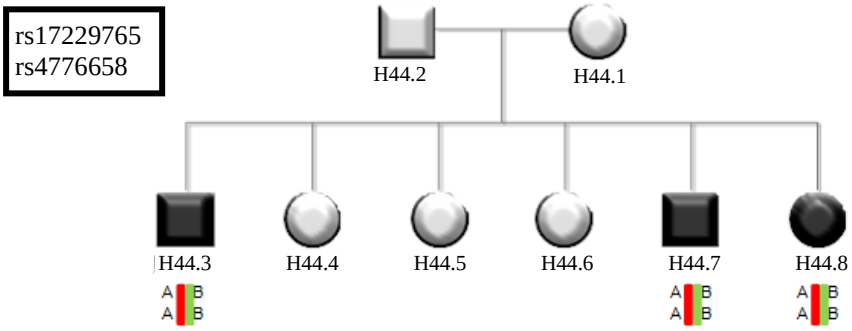


Figure F.7. Haplotype of the family H44 for the markers spanning the SPG21 locus. The box on the left shows the order of the SNP markers used for this locus.

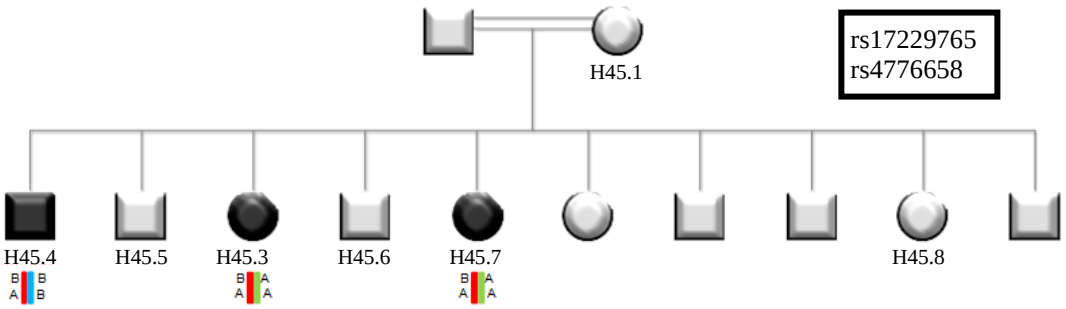


Figure F.8. Haplotype of the family H45 for the markers spanning the SPG21 locus. The box on the right shows the order of the SNP markers used for this locus.

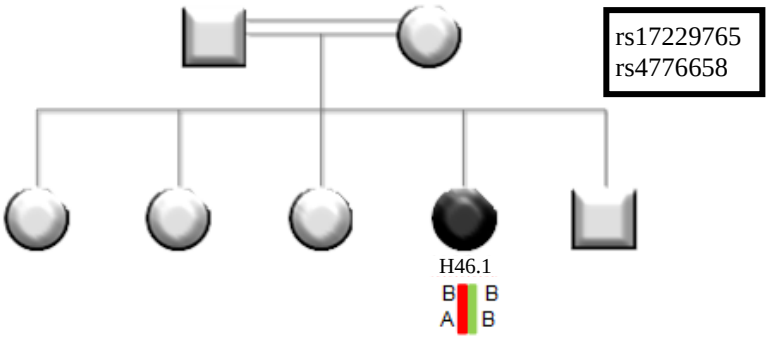


Figure F.9. Haplotype of the family H46 for the markers spanning the SPG21 locus. The box on the right shows the order of the SNP markers used for this locus.

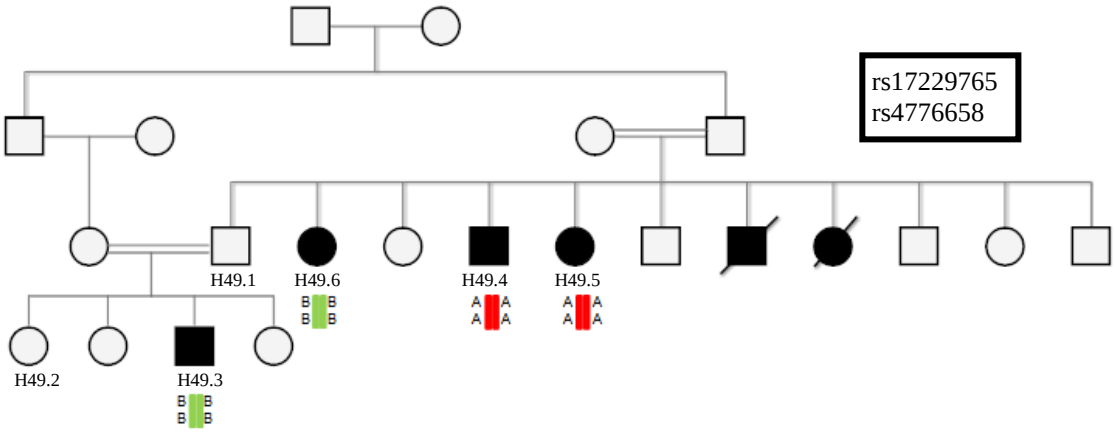


Figure F.10. Haplotype of the family H49 for the markers spanning the SPG21 locus. The box on the right shows the order of the SNP markers used for this locus.

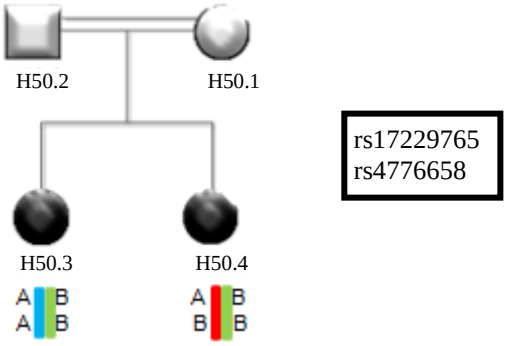


Figure F.11. Haplotype of the family H50 for the markers spanning the SPG21 locus. The box on the right shows the order of the SNP markers used for this locus.

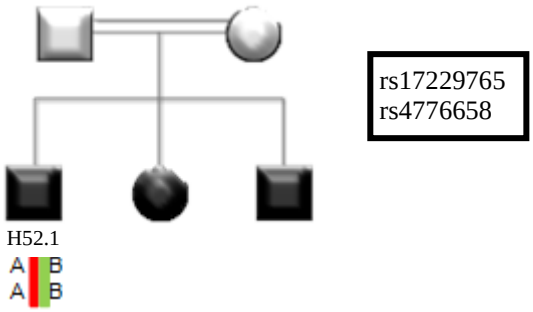


Figure F.12. Haplotype of the family H52 for the markers spanning the SPG21 locus. The box on the right shows the order of the SNP markers used for this locus.

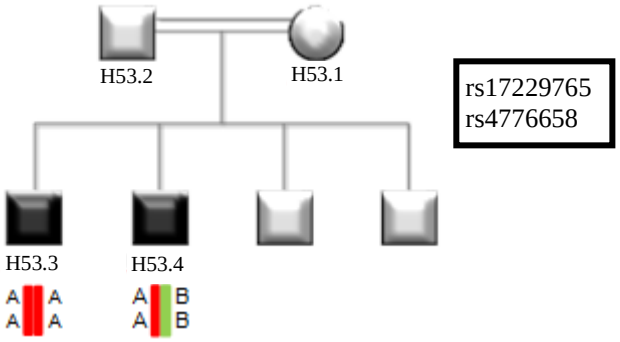


Figure F.13. Haplotype of the family H53 for the markers spanning the SPG21 locus. The box on the right shows the order of the SNP markers used for this locus.

APPENDIX G: LOD SCORE ANALYSES

LOD score results were calculated by SuperLink 1.6 for family H29 and by FastLink 4.1 and SuperLink 1.6 for family H45. Maximum LOD score was calculated by FastSLink 2.51 for both families. These programs were run on EasyLINKAGE 5.08 program. LOD score results are given in Figure G.1 through Figure G.5.

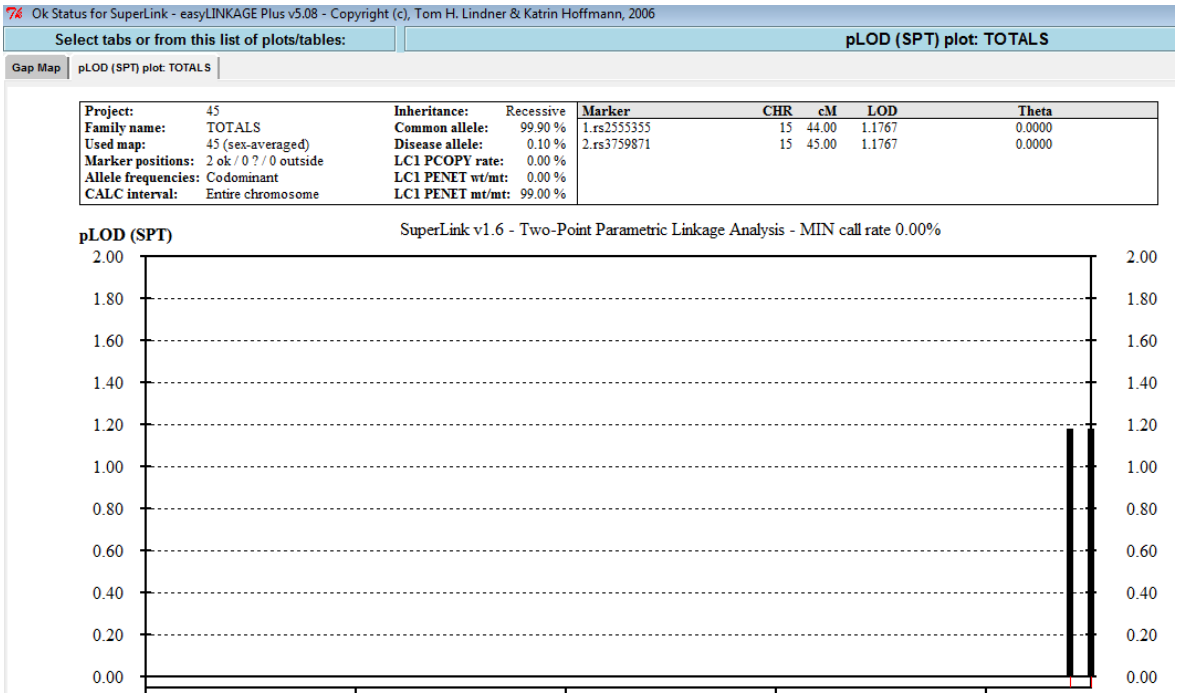


Figure G.1. LOD score result for family H45 calculated by SuperLink 1.6 run on EasyLINKAGE 5.08 program.

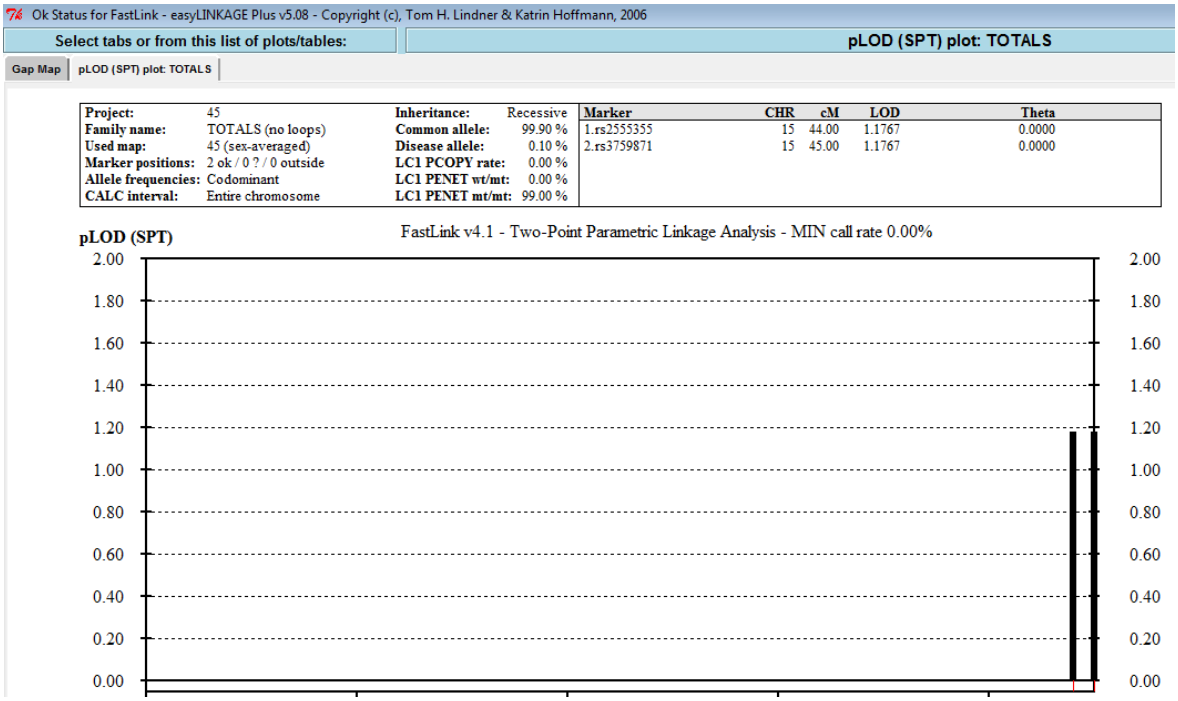


Figure G.2. LOD score result for family H45 calculated by FastLink 4.1 run on EasyLINKAGE 5.08 program.

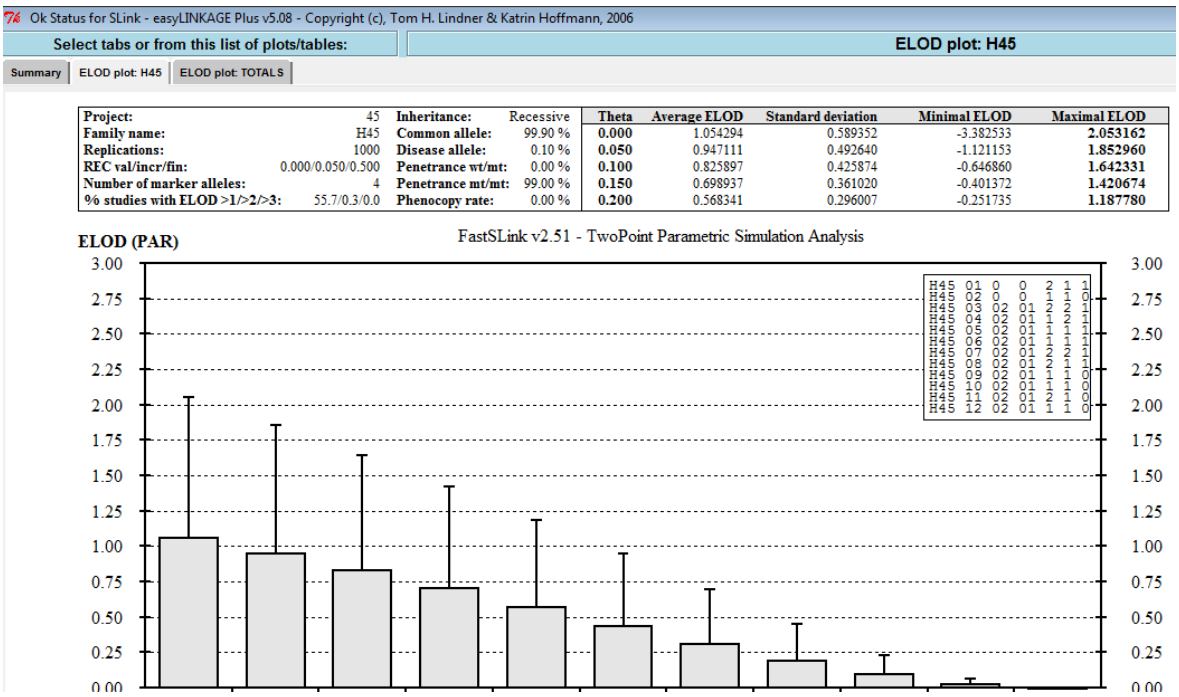


Figure G.3. Maximum LOD score result for family H45 calculated by FastSLink 2.51 run on EasyLINKAGE 5.08 program.

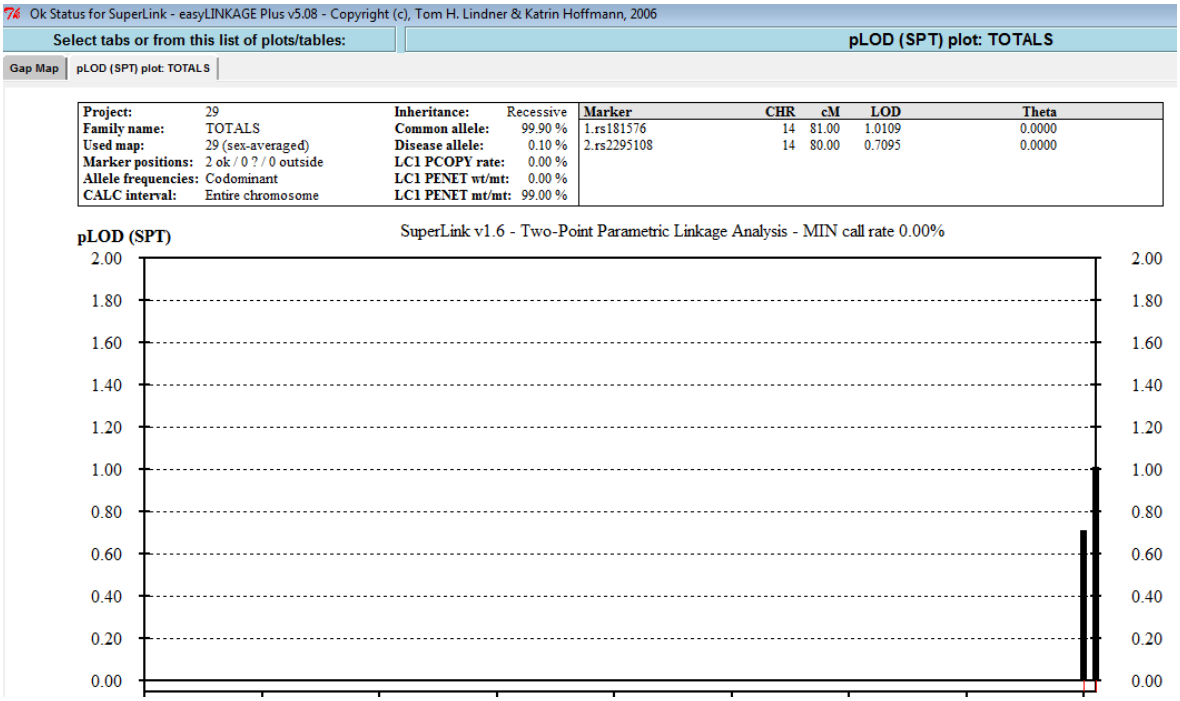


Figure G.4. LOD score result for family H29 calculated by SuperLink 1.6 run on EasyLINKAGE 5.08 program.

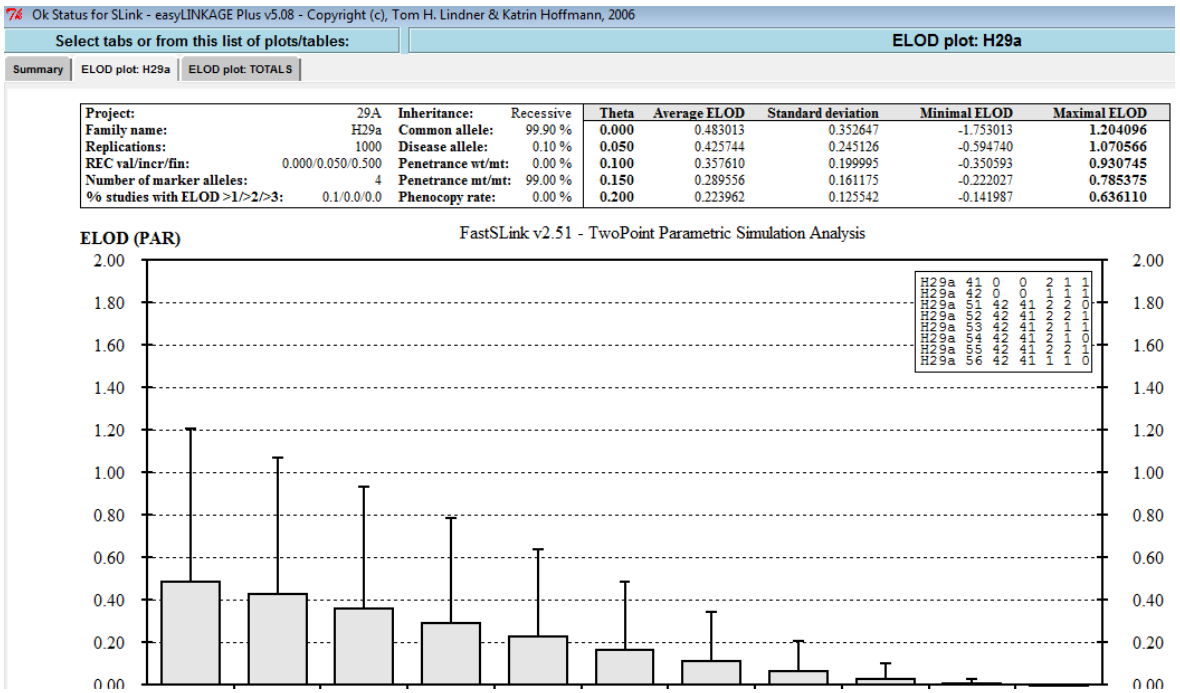


Figure G.5. Maximum LOD score result for family H29 calculated by FastSlink 2.51 run on EasyLINKAGE 5.08 program.

APPENDIX I: DIRECT SEQUENCING RESULTS

Direct sequencing results for patient H49.5 for six exonf of the CYP7B1 gene are given in the figure I.1 through I.5. Sequence alterations were not identified in this patient.

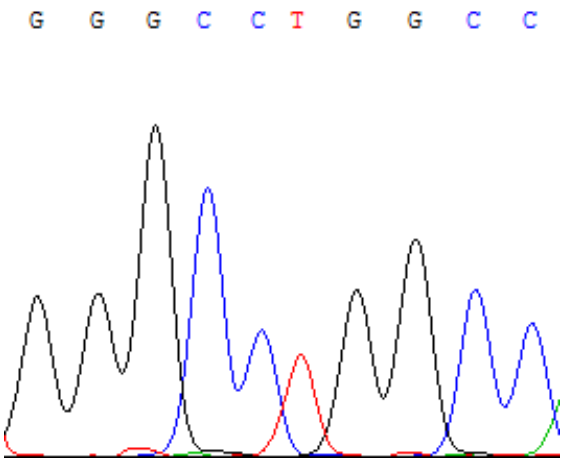


Figure I.1. Partial chromatogram displaying (59th – 69th bases in the coding sequence) exon one of CYP7B1 gene in patient H49.5 in the sense strand.

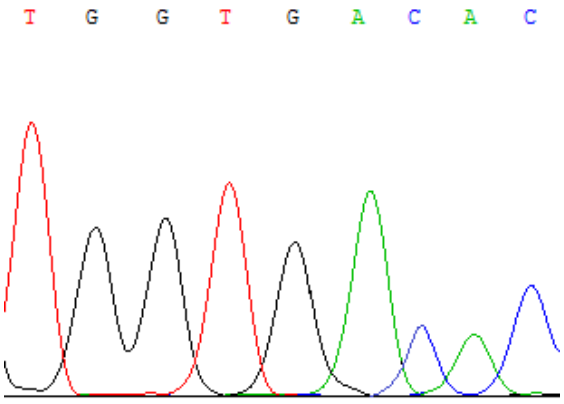


Figure I.2. Partial chromatogram displaying (109th – 117th bases in the coding sequence) exon two of CYP7B1 gene in patient H49.5 in the sense strand.

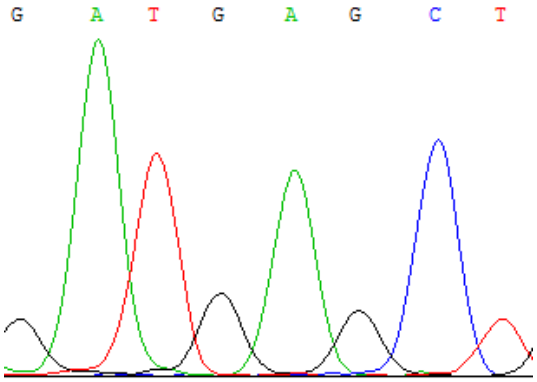


Figure I.3. Partial chromatogram displaying (146th – 154th bases in the coding sequence) exon three of CYP7B1 gene in patient H49.5 in the sense strand.

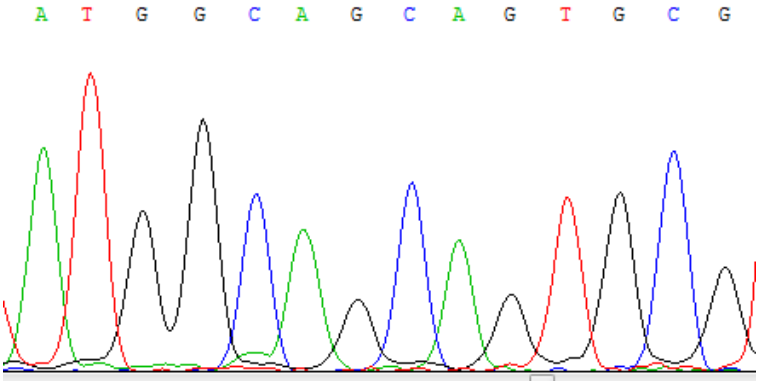


Figure I.4. Partial chromatogram displaying (93rd – 106th bases in the coding sequence) exon four of CYP7B1 gene in patient H49.5 in the sense strand.

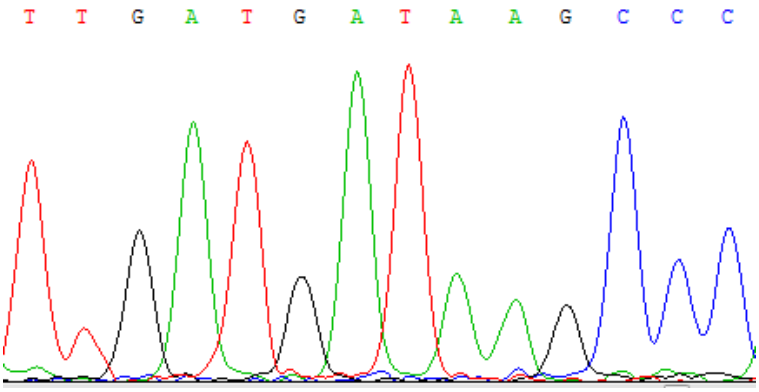


Figure I.5. Partial chromatogram displaying (191st – 204th bases in the coding sequence) exon six of CYP7B1 gene in patient H49.5 in the sense strand.

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