GENETICS AND MECHANISMS OF ESSENTIAL TREMOR AND RELATED DISORDERS

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By

Hilal Ünal Gülsüner

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We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Doctor of Philosophy.

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ABSTRACT

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Ph.D. in Materials Science and Nanotechnology

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Neurodegenerative disorders are characterized by progressive nervous system dysfunction, and they remain as one of the most challenging disorders known to humankind. These disorders have devastating effects on patients and currently there are no effective therapeutic approaches. Approved medicines provide only symptomatic relief, and inadequacy of information about the molecular mechanisms underlying these conditions restricts the development of new effective therapies. In this thesis, I presented the genetic analysis of two different neurodegenerative disorders and investigate the molecular mechanisms underlying these two disorders: essential tremor and Troyer syndrome. Essential tremor is one of the most prevalent movement disorders; however, its genetic cause and molecular mechanisms remain unknown because of its clinical heterogeneity, age-dependent penetrance, variable expressivity, and relation to other neurodegenerative disorders. In a six-generation consanguineous Turkish family with both essential tremor and Parkinson's disease, we identified a rare missense mutation of *HTRA2* as the causative allele. Family members homozygous for this allele were more severely affected than those heterozygous for this allele. Troyer

syndrome is a very rare autosomal recessive neurodegenerative disorder with only several described cases. Only two reports with truncating mutations have been described. In a consanguineous Turkish kindred with two affected siblings presenting tremor of the hands as well as clinical features similar to that of Troyer syndrome, we identified a novel missense mutation in *SPG20*. We presented a genotype-phenotype correlation in this family, and the missense SPG20 p.G580R mutation was found to result in a milder form of Troyer syndrome without skeletal abnormalities. Overall, this study provides solutions to complexities of neurodegenerative disorders by suggesting a novel and unifying molecular mechanism underlying essential tremor and Parkinson's disease. Furthermore, correlation of genotypes and phenotypic differences in patients with Troyer syndrome explains the clinical heterogeneity and variable expressivity of neurodegenerative disorders.

Keywords: Disease gene identification, Essential tremor, hereditary movement disorders, mutation, next-generation sequencing, Troyer syndrome.

ÖZET

ESANSİYEL TREMOR VE BENZERİ HASTALIKLARIN GENETİĞİ VE MEKANİZMASI

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Nörodejeneratif hastalıklar ilerleyici sinir sistemi bozuklukları olarak tanımlanırlar ve insanlık tarihinde karşılaşılan en zorlu hastalıklardır. Hastalar üzerindeki etkileri çok tahrip edici boyutlara ulaşabilen nörodejeneratif hastalıkların etkili bir tedavi yöntemleri bulunmamaktadır. Günümüzde kullanılan tedavi yaklaşımları sadece bu hastalıkların belirtilerini azaltmaya yöneliktir. Günümüzde bu tür hastalıkların altında yatan moleküler mekanizmalar ile ilgili yeterli bilgiye sahip olunmaması, yeni ve etkili tedavi yöntemlerinin geliştirilebilmesi konusunda çok büyük engel teşkil etmektedir. Bu çalışmada iki farklı nörodejeneratif hastalık olan esansiyel tremor ve Troyer sendromunun genetik incelemeleri sunulmuştur. Esansiyel tremor en sık görülen hareket bozukluklarından biri olmasına rağmen, bu hastalığın klinik heterojenliği, yaşa bağlı penetransı, değişken ifade edilmesi ve diğer nörodejeneratif hastalıklar ile olan benzerlikleri nedeniyle genetik ve moleküler kökenleri henüz tam olarak tespit edilememiştir. Bu çalışmada, Türkiye'de yaşayan, esansiyel tremor ve Parkinson hastalarının görüldüğü ve akraba evliliği olan 6 kuşaklık bir aile tanımlanmıştır. Bu ailedeki 3 hastadan alınan DNA ile gerçekleştirilen tüm ekzom dizilemesi sonucunda

HTRA2 geninde hastalık yapıcı bir yanlış anlamlı mutasyon tespit edilmiştir. Bu mutasyonu homozigot olarak taşıyan kişilerde hastalık belirtileri heterozigot bireylere göre çok daha şiddetlidir ve bu bireylerde orta yaştan itibaren Parkinson hastalığı gelişmiştir. Troyer sendromu ise çok nadir görülen otozomal çekinik bir hastalıktır. Bu hastalığın nedeni olarak şu anda kadar sadece *SPG20* geninde protein ifadesini yok eden iki farklı çerçeve kayması mutasyonu tanımlanmıştır. Bu çalışmada, el titremesi ile birlikte Troyer sendromu belirtilerini taşıyan ve akraba evliliği sonucu dünyaya gelmiş iki kardeş tanımlanmıştır. Bu iki hasta bireyden izole edilen DNA ile gerçekleştirilen tüm ekzom dizileme çalışmaları sonucunda *SPG20* geninde yeni bir yanlış anlamlı mutasyon keşfedilmiştir. Bu ailede saptanan SPG20 p.G399S mutasyonu, tanımlanan diğer Troyer vakalarından farklı olarak, iskelet anomalilerine neden olmamaktadır. Bu çalışmalar esansiyel tremor ve Parkinson hastalıklarında yeni ve birleştirici bir mekanizma sunarak, nörodejeneratif hastalıkların karmaşık yapılarına bir çözüm getirmektedir. Ayrıca, Troyer sendromlu ailede tanımladığımız fenotip şiddeti ile genotip arasında saptanan ilişki, nörodejeneratif hastalıkların klinik heterojenliği ve değişken ifadelerini aydınlatmaktadır.

Anahtar sözcükler: Esansiyel tremor, hastalık gen tanımlanması, kalıtsal hareket bozuklukları, Troyer sendromu, yeni nesil dizileme.

Annem, Babam ve Kardeşim'e

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HİLAL ÜNAL GÜLSÜNER

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ABBREVIATIONS

AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
CDK5	Cyclin dependent kinase 5
ET	Essential Tremor
HSP	Hereditary spastic paraplegia
HTRA2	High temperature requirement protein A2
IBD	Identity by descent
NGS	Next generation sequencing
PD	Parkinson's disease
PINK1	PTEN induced putative kinase
ROS	Reactive oxygen species
SPG20	Spastic paraplegia 20
TS	Troyer syndrome

URLs

1000 genomes project	1000genomes.org
GATK	broadinstitute.org/gatk/
Mutation Assessor	mutationassessor.org
NHLBI exome sequencing project	evs.gs.washington.edu/EVS/
Polyphen-2	genetics.bwh.harvard.edu/pph2/
SIFT	sift.jcvi.org/

Chapter 1

1 Introduction

1.1 Neurodegenerative Disorders

Neurodegeneration is the loss of the structure and death of the neurons in motor, sensory or cognitive systems. Neurodegenerative disorders are characterized by progressive nervous system dysfunction which begins insidiously and follow a slow but progressive course. The level of neurodegeneration can range from molecular to cellular levels. Individuals first have mild symptoms like problems in coordination or memory. However, as cells start to deteriorate, symptoms progressively get worse, such as patients might lose their ability to walk, move, or think clearly.

There are more than a few hundreds of neurodegenerative disorders. Most of them overlap with each other clinically and pathologically. Neurodegenerative disorders can be classified depending on the pattern of cell loss in different regions of the brain. They are grouped into the basal ganglia, brainstem and cerebellum, cerebral cortex, or spinal cord disorders (1) (Table 1). Basal ganglia disorders are characterized by abnormal movements or defects in the initiation of motor function (2). Parkinson's disease, which is characterized by loss of dopaminergic neurons and presence of Lewy bodies, is a hypokinetic basal ganglia disorder; that is, higher than normal basal ganglia output causes inhibition of thalamocortical neurons and reduces motor function (2). Huntington's disease is characterized by chorea and is a hyperkinetic basal ganglia disorder. It is caused by reduced basal ganglia output, which increases thalamocortical

function and uncontrollable motor function (2). Essential tremor, Friedreich's ataxia, multiple systems atrophy, and spinocerebellar ataxia are examples of brainstem and cerebellum disorders. Classification of diseases that predominantly affect brainstem and cerebellum is especially challenging because of the overlap among pathological symptoms. Cerebral cortex is cerebrum's outer layer and plays a key role in memory, language, attention, perceptual awareness, and consciousness (3). One of the disorders that occur due to damage in cerebral cortex is Alzheimer's disease which is characterized with neuropathological features of neuronal loss, senile plaques, neurofibrillary tangles and acetylcholine deficiency. Diffuse Lewy body disease and Pick's disease are other examples of cerebral cortex disorders. Spinal cord supports nerve cells that extend from medulla oblongata to brainstem. Defects in spinal cord destroy motor neurons which function in voluntary muscle activities such as speaking, walking, breathing and swallowing (3). The neurodegenerative diseases with defective spinal cord are hereditary spastic paraplegia, amyotrophic lateral sclerosis, and spinal muscular atrophy.

Central nervous system diseases remain one of the most challenging disorders known to humankind. Neurological disorders have devastating affects to patients and there is no effective therapeutic approach. Currently, medications can only provide symptomatic relief, and the development of new effective therapies is prevented by the limited knowledge of the molecular mechanisms underlying these conditions.

Table 1.1 Clinical and pathological features of hereditary neurodegenerative disorders and the list of mutated genes

Disease and Predominantly Affected Site	Neuropathological Features	Mutated Genes*
Basal Ganglia		
Huntington's disease	neuronal loss in neurostriatum and cerebral cortex	HTT
Parkinson's disease	neuronal loss in substantia nigra, Lewy bodies in pigmented neurons	EIF4G1, LRRK2, PARKIN, PARK7, PINK1, SNCA, VPS35
Brain stem and Cerebellum		
Essential tremor	large Bergmann glial cells and torpedoes in cerebellum	FUS, DNAJC13
Friedreich's ataxia	neuronal loss in motor and sensory systems	FXN
Multiple system atrophy	neuronal and glial inclusions	SNCA
Spinocerebellar ataxia	neuronal loss in cerebellum and brainstem	Multiple genes (e.g. ATXN1, ATXN2, ATXN3, C10ORF2)
Cerebral Cortex		
Alzheimer's disease	senile plaques, neurofibrillary tangles, neuronal and synaptic loss	APP, PSEN1, PSEN2
Pick's disease	frontotemporal atrophy, pick bodies	NPC1, NPC2, SMPD1
Spinal cord		
Amyotrophic lateral sclerosis	loss of upper and lower motor neurons, Bonina bodies and axonal spheroids	ANG, ALS2, C9ORF72, FIG4, FUS, OPTN, SETX, SOD1, SPG11, TARDBP, UBQLN2, VAPB, VCP
Hereditary spastic paraplegia	loss of lower motor neurons	Multiple genes (e.g. ATL1, KIAA0196, PLP1, SPG7, SPG11, SPG20, SPAST, ZFYVE26)
Spinal muscular atrophy	loss of lower motor neurons	DYNC1H1, SMN1, SMN2, UBA1, VAPB

*Only established Mendelian disease genes are listed, susceptibility loci are not included.

1.2 Genetics and Mechanisms of Neurodegenerative Disorders

In addition to their extensive clinical diversity, most of the neurodegenerative disorders are genetically heterogeneous. However, the insights gained through genetic studies reveal some overlapping mechanisms (4). These include protein aggregation, oxidative stress, excessive reactive oxygen species formation, mitochondrial dysfunction, energy depletion and autophagy disruption, endoplasmic reticulum stress, neuroinflammation, and several environmental factors.

1.2.1 Protein misfolding and aggregation

Proteins form networks based on their physical interactions and these networks determine every aspect of life to ensure that cells and organisms function properly. Proteins must maintain their three dimensional structures to stay functionally active in such networks. Chaperone proteins organize protein folding in order to prevent mistakes and malfunctioning proteins. However, partial protein folding or misfolding, aggregation and deposition have been observed in the brain tissues of neurodegenerative disorders. These disorders are classified as protein conformational diseases or proteinopathies (5). Some examples of proteinopathies are CAG trinucleotide repeat expansion diseases such as Huntington's disease and spinocerebellar ataxias (6). Parkinson's disease, amyotrophic lateral sclerosis, prion disease, and Alzheimer's disease are also classified as proteinopathies (6). The most important feature of these proteinopathies is that a certain protein folds into an alternative stable conformation which results in its aggregation in the tissues (6). Examples of the aggregated proteins are α-synuclein (encoded by *SNCA*) in Parkinson's disease, amyloid-β (cleaved from

amyloid-β precursor protein (APP) with the help of enzymes encoded by presenilin1 (*PSEN1*) and presenilin 2 (*PSEN2*)) in Alzhemier disease, huntingtin (encoded by HTT) in Huntington's disease and superoxide dismutase (encoded by *SOD1*) in amyotrophic lateral sclerosis (6).

The link between protein aggregation and neurodegeneration is not well understood. The hydrophobic patches of these aggregated proteins might interact with other components of the cell and cause damage. In addition, these proteins are part of essential cellular networks, including chromatin organization, vesicular transportation, transcription and translation, cell architecture and protein quality control (7). Therefore, their misfolding and aggregation results in disintegration of these crucial cellular functions (7).

There are several systems used by eukaryotes to remove the malfunctioning proteins and to prevent their aggregation. Cells have protein quality control pathways in order to avoid defects in protein homeostasis. The protein homeostasis is regulated by molecular chaperones, autophagy and ubiquitin proteosome system (UPS) (PARKIN, which is mutated in AD, involves in UPS) to clear proteins expressed in abundance. However, these protein control mechanisms become overwhelmed with age by several genetic and environmental factors. Therefore misfolded proteins can contribute to neurodegenerative disorders by loss-of-function of the protein aggregates, gain-of-toxic-activity, or by causing brain inflammations (6).

1.2.2 Mitochondrial dysfunction and oxidative stress

Mitochondria are present in all cells of the body except erythrocytes and range from hundreds to thousands in number. They provide an organism's housekeeping

functions, including production of the adenosine triphosphate (ATP), biosynthesis of aminoacids, beta-oxidation of fatty acids, and apoptosis (4). Due to their high need of bioenergetics, mitochondria are enriched at presynaptic and postsynaptic terminals of neurons (4).

One of the most common mitochondrial defects that lead to neurodegeneration is oxidative stress. Mitochondria produce ATP through the oxidation of metabolic intermediates generated during Krebs cycle (8). ATP is generated by electron transport chain (ETC) via redox reactions across the inner mitochondrial membrane. Cells can defend themselves against those reactive oxygen species (ROS), produced during redox reactions, by several antioxidant enzymatic activities including superoxide dismutase and glutathione peroxidase; or scavengers including ascorbic acid (vitamin C), β-carotene, ubiquinone, lipoic acid and tocopherol (vitamin E) (9). However, disturbances in the regular redox state of the cells might cause increased ROS. The resulting increased ROS production and insufficiency of antioxidant defense mechanism is called oxidative stress (10). Neuronal tissue is particularly more vulnerable to oxidative stress and damage; moreover, brain contains relatively low levels of antioxidants which lead to neurodegeneration (10). For example, *PINK1* encodes a serine/threonine kinase located in mitochondria. Mutations in this gene causes early onset Parkinson's disease due to stress induced degeneration of dopaminergic neurons (11).

Beside oxidative stress, other major defects in mitochondria that lead to neurodegeneration are abnormal mitochondrial trafficking, distorted inter-organellar communication, and damaged mitochondrial quality control (4). SOD1 mutations induce mitochondrial membrane damage, discharging mitochondrial membrane potential and leading to ALS (11). Several other neurodegenerative diseases including

Parkinson's disease, Alzheimer's disease and Huntington's disease are known to be caused by alterations or dysfunction in mitochondrial dynamics (12).

1.2.3 Endoplasmic reticulum stress

Endoplasmic reticulum (ER) is an important organelle for protein synthesis, folding, post-translational modification and transport (13). Accumulation of misfolded proteins and changes in calcium homeostasis lead to ER stress due to the loss of ER integrity or normal function. In order to restore the effects of ER stress, unfolded protein response (UPR) is activated by changing the expression of ER chaperones and enhancing degradation of misfolded or aggregated proteins. Sustained ER stress can lead to the activation of cell death pathways (13).

The defects in the cellular processes involving ER cause different degrees of ER stress contributing to neurodegenerative disorders such as Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease, PolyQ diseases such as Huntington's disease, and transmissible spongiform encephalophaties (TSE), or prion disease (13-18).

1.2.4 Axonal transport deficits

Motor neurons are large polarized cells. Their function is dependent on the intracellular transport of molecules along their axons. Anterograde axonal transport (towards the axon tip) carries proteins and lipids to the distal synapse and mitochondria (19). Retrograde transport (away from the synapse towards the soma) is essential for the clearance of defective proteins from the axon (19). Kinesin and dynein complexes are responsible for the axonal transport of cargoes in the anterograde and retrograde direction, respectively.

Defects in axonal transport are common factors in many neurodegenerative disorders. In Alzheimer's disease, mutant presenilin 1 and amyloid-β impairs cargo attachment to kinesins and damages axonal transport (20, 21). Mutations in heat shock factor binding protein 1 (HSBP1) cause microtubule deacetylation and disruption of neurofilament assembly resulting in Charcot-Marie-Tooth disease (22). More than 40% of pure hereditary spastic paraplegias (HSPs) occur due to mutations in spastin (SPG4) which results in a progressive axonopathy affecting the cortiospinal tract (23). In ALS, mutations in dynactin 1 (DCTN1) protein harm microtubule-based axonal transport (24). The most common cause of ALS occurs due to the mutations in superoxide dismutase (SOD1). Mutant SOD1 together with glutamate inhibits cargo from binding to the motor protein which slows axonal transport (25).

1.2.5 Environmental factors

Acute or chronic exposure to environmental toxins can also cause neurodegeneration. Lead poisoning is one of the most common examples which cause myelin loss and axonal degeneration. Other metals (mercury, aluminum, zinc), pesticides, electromagnetic fields, brain injuries, inflammation, lifestyle and dietary factors are also associated with the increased risk of neurodegenerative disorders (26).

Currently, there is no cure for neurodegenerative diseases, but the medications aim to ease the symptoms. The advances in technology allow the identification of new disease causing genes every day, which results in the exploration of common pathways that lead to certain neurological disorders. This will allow the development of new targeted therapeutic approaches for these pathways.

1.3 Disease Gene Identification Strategies

As discussed in section 1.2., neurological disorders are influenced by both defective genes and environment. Identification of genes responsible for diseases is the first step to understand the human genome, to understand the pathophysiology of the disease, and the role of underlying proteins and pathways (27).

1.3.1 Traditional Methods

The most commonly used technique for the identification of Mendelian disease genes have been positional mapping through linkage analysis (28). Prior biological or medical knowledge is not needed to perform linkage analysis; therefore it is unbiased. The theory behind linkage analysis was developed by Thomas Morgan in 1910 that traits that are co-inherited are likely to be due to "factors" that are "linked" (29). Genes located in close proximity on a chromosome are less likely to have recombination events between them, and they tend to co-segregate together. Based on this, the first genetic map to estimate the distance between trait-causing genes based on recombination was created. If the two traits are co-segregated in a family, then they are assumed to be linked (30). Logarithm of odds (LOD) analysis compares the probability of obtaining the observations if the two loci are linked, to the probability of observing the same data by chance (30). For Mendelian disorders, a LOD score greater than 3 suggests linkage. In 1977 spinocerebellar atrophy (SCA1) became the first neurolodegenerative disorder linked to a chromosome (HLA region of chromosome 6) (31). Later, in 1983, Huntington's disease became the first neurologic disorder mapped to a genomic locus using polymorphic DNA markers (32). Although this technology allowed the identification of ~3,000 Mendelian disease loci (28), this approach yields only regions of linkage and not the causative gene.

The discovery of DNA sequencing through chain termination of dideoxynucleotides (ddNTPs) by Frederic Sanger in 1977 was a breakthrough in genomic technologies. This approach allowed the sequencing of candidate genes identified through positional mapping, and had been the most widely used sequencing technique for approximately 30 years (33). Nevertheless, identification of the genes present in the mapped region required several labor-intensive steps, such as cloning the region, and then determining the disease causing gene. For example, although Huntington's disease was the first neurodegenerative disorder mapped to a certain locus, it took 10 years of research to clone the region and identify the disease causing gene (34). Medium-throughput DNA sequencing through automated capillary electrophoresis allowed the identification of human reference genome during the Human Genome Project from 1990 to 2001. This development eased the identification of disease causing mutations by eliminating several steps of cloning that required several years of research. However, even with the human reference sequence, most of the disease causing genes remained elusive due to large linkage regions containing hundreds of genes, which increased the costs of follow-up sequencing projects (35).

In addition to linkage analysis, homozygosity mapping is another powerful method to identify recessive disease genes by identifying homozygous regions shared by the affected family members when the family is either consanguineous or practice endogamy (36). This technique relies on genome wide single nucleotide polymorphism (SNP) arrays of affected and unaffected family members.

Despite these advances in technology, a particular category of diseases remained largely unsolved: rare genetic disorders due to the limited number of family members,

or diseases with *de novo* events, locus heterogeneity, reduced penetrance or variable expressivity.

1.3.2 Whole Exome Sequencing

Since 2005, next generation sequencing (NGS) platforms become available and reduce the cost and time of whole genome sequencing. NGS platforms are also applied to target the protein coding regions of the genome (known as the "exome") through sequence capture by hybridization and massively parallel sequencing (Figure 1.1A). This allows the sequencing of the best known ~1% of the human genome which is estimated to hold ~85% of the mutations associated with disease phenotypes (28). Currently, the cost of whole exome sequencing technology is reduced to ~$600. Discovery of Mendelian disease genes through exome sequencing has been growing exponentially since it was first demonstrated in 2009 (37). Ongoing gene identification reveals genetic causes underlying neurological disorders and illuminates new biochemical pathways.

Whole exome sequencing has successfully been applied for the identification of disease causing mutations in rare Mendelian diseases such as brain malformations, many forms of cerebellar ataxia and spastic paraplegia (38-40). Along with rare Mendelian diseases, whole exome sequencing has also been applied to common and complex disorders to identify highly penetrant variants in familial forms of Parkinson's disease, Alzheimer's disease, essential tremor, and multiple sclerosis (41-44).

The main steps required for whole exome sequencing are shown in Figure 1.1A. Genomic DNA is randomly sheared into 200-300 bp by sonication. The ends of the fragments are converted into blunt ends by removing 3' overhangs and filling 5'

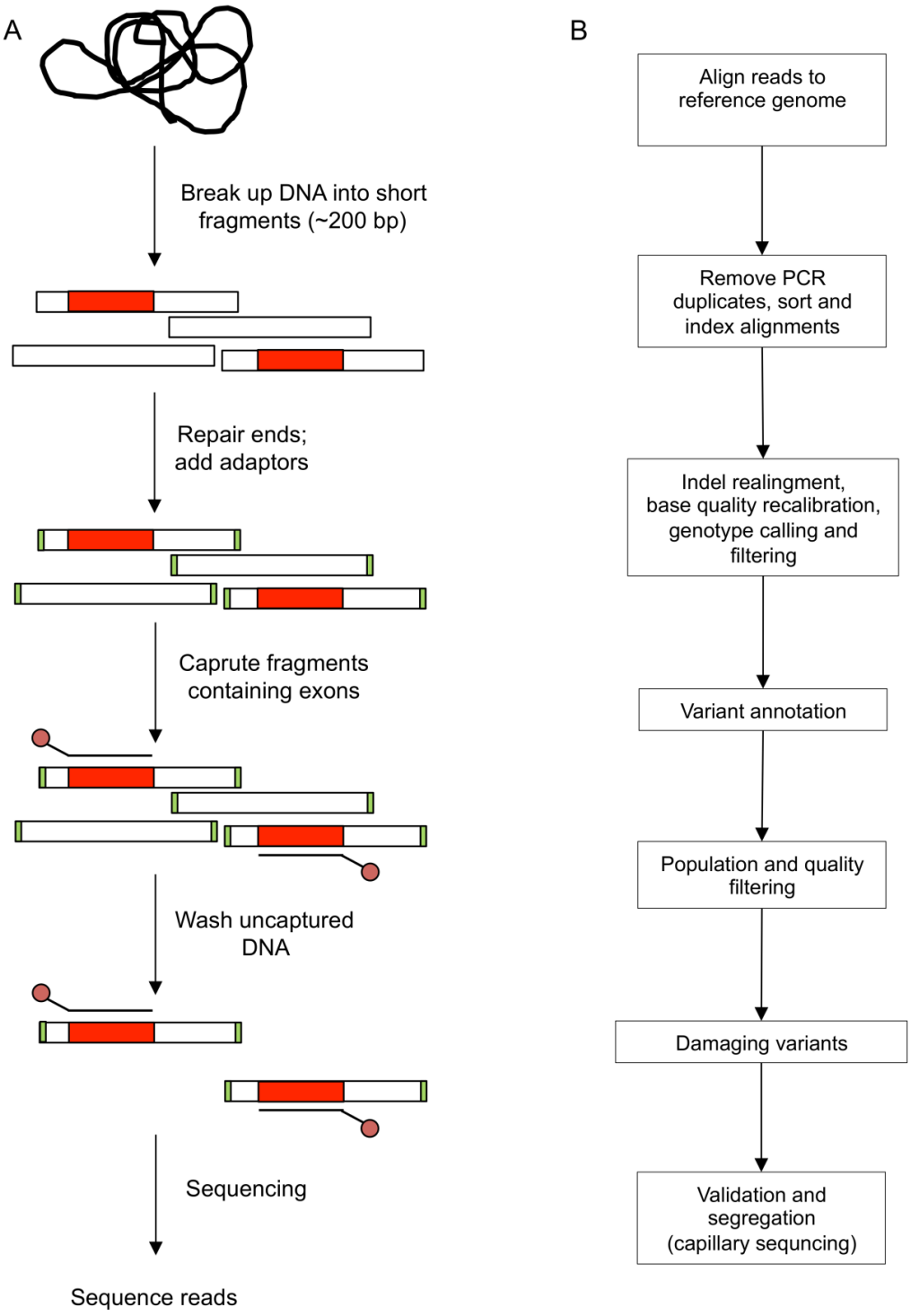

Figure 1.1 Workflow of exome sequencing. *(A)* Isolated genomic DNA is randomly sheared into 200-300 bp followed by end repair, A-tailing and indexed paired-end adapter ligation. Exomes are captured and hybridized to biotinylated capture probes, and finally libraries are sequenced. (Copyright 2012 Nature Publishing Group. From Bras et al (35) with permission) *(B)* Flowchart of exome data analysis and variant filtration.

overhangs. Then the 3' ends of the fragments are adenylated (A-tailing). Corresponding thymine nucleotide on the 3' end of the adapters is ligated to the complementary fragment. DNA fragments with adapter on both ends are amplified by PCR. The resulting exome library is hybridized to biotinylated DNA beads. The captured DNA is amplified by PCR and is sequenced (Figure 1.1A).

Whole exome sequencing generates millions of short sequence reads per run and the main challenge of whole exome sequencing is to make sense of the large amount of data. Identification of disease genes requires several steps of analytical and functional filtering and analyzing the data to sort through thousands of variants that differ from the human reference sequence (Figure 1.1B). The first analytical step after receiving whole exome sequence data is to map the millions of short sequences to a reference human genome. This is obtained from the reference assembly formed by the Human Genome Project. In order to detect the regions differ from the reference human genome, overlapping reads are required with a minimum coverage of 8 reads for heterozygous genotype calls and 4 reads for homozygous calls. A minimum of 30 reads per base is generally needed throughout the genome to reach the ideal depth of sequencing. Since sequencing platforms have error rates, it is important to differentiate the true variants from sequencing artifacts (45). In order to detect the variants that are rare or private to the subject, variants present in public databases (dbSNP, 1000genomes, EVS, or a set of unaffected individuals) are filtered out. During this filtration, the threshold for minor allele frequency (MAF) should be set accordingly. The next step is the functional filtering where detection of the functional variants among neutral coding variants is needed. Nonsense mutations and frameshift insertions and deletions resulting in a premature stop codon generally lead to truncated and usually nonfunctional protein products. In addition, several computational tools are used to predict the impact of

missense variants on protein function, such as SIFT, PolyPhen2, and MutationAssessor, in order to distinguish pathogenic variants from neutrals (46-48). These tools use biochemical properties of amino acid changes and multiple sequence alignments to obtain the degree of conservation among aminoacid residues in closely related sequences. Finally the candidate variants are verified by genotyping or capillary sequencing, and segregation status is analyzed if the family members are available.

Now exome sequencing becomes the *de facto* approach to identify disease causing mutations. Although whole exome sequencing has increased the disease gene identification rate, there are still some major reasons why one can not find a variation associated with the disease. These are the lack of or poor sequence coverage of the coding regions, false negative calls in regions with poor quality sequence data, mutations in non-coding regions, and misinterpretation of missense variants (49). Whole genome sequencing and new prediction methods have been used to increase the success rate of disease gene identification.

1.4 Consanguinity

Individuals related as second cousins or closer are defined as consanguineous (50). The most common consanguineous marriages occur between first cousins. Since spouses share 1/8 of their genome, their progeny are homozygous for 1/16 of all loci. Consanguineous marriages are culturally favored in some countries, such as North Africa, West Asia and South India, and constitute around 20-50% of all marriages (51) (Figure 1.2). Consanguineous marriage rate in Turkey is around 20%.

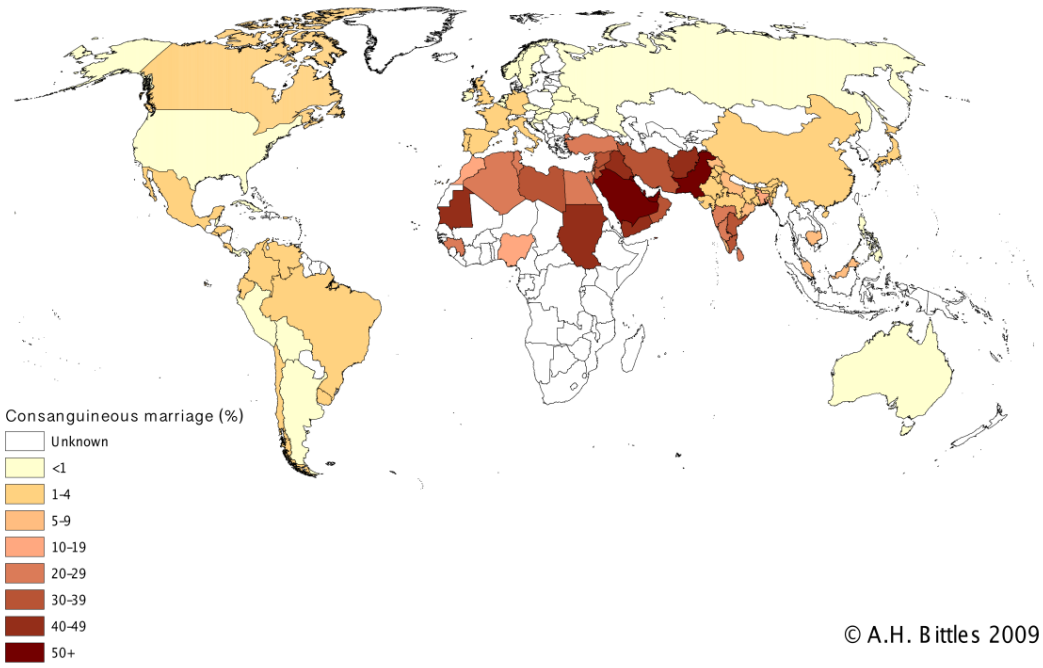

Figure 1.2 Global prevalence of consanguinity. (http://www.consang.net with permission from A.H. Bittles)

Consanguineous marriages have detrimental health effects as it increases the probability of disease causing mutations to reside in homozygous regions (52). Consanguineous populations have limited gene pool by founder effect; moreover, the risk of observing recessive phenotypes due to two copies of the same mutation is increased. A genomic region is classified as identical by descent (IBD) if contiguous SNPs share the same genotype in the family members. The IBD regions shared by all affected individuals that differ from the unaffected individuals are considered as disease causing region. Availability of high-density genome-wide SNP genotyping panels ease to trace IBD regions by analyzing the shared homozygous regions in consanguineous families with recessive disorders. (53). This can lead to the identification of founder mutations in isolated populations. In addition, consanguineous marriages may also introduce individuals with common disorders with a much severe phenotype due to

homozygosity; therefore, allows the identification of causative dominant mutations with dosage effect (54).

1.5 Outline of the Thesis

In this dissertation, my work on the genetic analysis of neurodegenerative disorders is divided into four chapters, including this introductory section.

The second chapter addresses my work on the analysis and characterization of a family segregating essential tremor and Parkinson's disease. Essential tremor is one of the most frequent movement disorders of humans, but its causes remain largely unknown. In a six-generation consanguineous Turkish family with both essential tremor and Parkinson's disease, we identified a rare missense mutation of *HTRA2* as the causative allele. Family members homozygous for this allele were more severely affected than those heterozygous for this allele. The same mutation had previously been associated with Parkinson's characteristics in mouse mutants and with Parkinson's disease in some, but not all, epidemiologic studies. Our results suggest that *HTRA2* might be responsible for essential tremor in some families and that homozygosity for damaging alleles of *HTRA2* may be responsible for Parkinson's disease.

The third chapter covers the genetic analysis of a family segregating Troyer syndrome. We identified a consanguineous Turkish kindred with two affected siblings presenting clinical features similar to that of Troyer syndrome. Affected siblings have a novel missense mutation in *SPG20*. Frameshift mutations in *SPG20* resulting into loss of function of spartin protein had previously been identified in Troyer syndrome patients. The clinical examinations showed that affected individuals in the Turkish family lack skeletal abnormalities observed in Amish and Omani populations with loss

of function mutations in *SPG20*. These findings suggest that truncated mutations in *SPG20* cause a much severe phenotype than that of missense mutations, which indicates a genotype phenotype correlation.

The final chapter summarizes my research and offers future perspectives on the genetic and molecular analysis of neurodegenerative disorders, and applications of these findings to clinical medicine.

Chapter 2

2 Mitochondrial serine protease HTRA2 p.G399S in a kindred with essential tremor and Parkinson's disease

The majority of the work described here was published in: Unal Gulsuner H., Gulsuner S., Mercan F.N., Onat O.E., Walsh T., Shahin H., Lee M. K., Dogu O., Kansu T., Topaloglu H., Elibol B., Akbostanci C., King M.-C., Ozcelik T., Tekinay A.B. "Mitochondrial serine protease HTRA2 p.G399S in a kindred with essential tremor and Parkinson disease", *Proc Natl Acad Sci USA*, 2014, doi: 10.1073/pnas.1419581111

2.1 Outline

Essential tremor is one of the most frequent movement disorders of humans and can be associated with substantial disability. Some but not all persons with essential tremor develop signs of Parkinson's disease, and the relationship between the conditions has not been clear. In a six-generation consanguineous Turkish kindred with both essential tremor and Parkinson's disease, we carried out whole exome sequencing and pedigree analysis, identifying HTRA2 p.G399S as the allele likely responsible for both conditions. Essential tremor was present in persons either heterozygous or homozygous for this allele. Homozygosity was associated with earlier age at onset of tremor ($P < 0.0001$), more severe postural tremor ($P < 0.0001$), and more severe kinetic tremor ($P =$

0.0019). Homozygotes, but not heterozygotes, developed Parkinson signs in the middle age. Among population controls from the same Anatolian region as the family, frequency of HTRA2 p.G399S was 0.0027, slightly lower than other European populations. *HTRA2* encodes a mitochondrial serine protease. Loss of function of *HtrA2* was previously shown to lead to parkinsonian features in motor neuron degeneration (*mnd2*) mice. HTRA2 p.G399S was previously shown to lead to mitochondrial dysfunction, altered mitochondrial morphology, and decreased protease activity; but epidemiologic studies of an association between *HTRA2* and Parkinson's disease yielded conflicting results. Our results suggest that in some families, HTRA2 p.G399S is responsible for hereditary essential tremor and that homozygotes for this allele develop Parkinson's disease. This hypothesis has implications for understanding the pathogenesis of essential tremor and its relationship to Parkinson's disease.

2.2 Introduction to Essential Tremor

2.2.1 Tremor in human history

Tremor is a rhythmic and oscillatory movement (55). This phenomenon was first recognized in ancient India (5000-3000 BC), Egypt (700 BC), and Greece (~400 BC) (56). There are also records of patients with kinetic and rest tremors in the writings of Galen of Pergamon (130-200 AD), Franciscus Sylvius (1680) and Gerard van Swieten (1745) (56). However, the first complete study was reported in 1887 by Dr. Charles Dana, with the presence of tremor in several large families (56).

2.2.2 Clinical features

Essential tremor (ET) is a progressive central nervous system disorder and is one of the most frequent movement disorders in humans (57). It is characterized by postural

or kinetic tremor of upper extremities, but head, legs, voice and other regions of the body may also be affected (58). Kinetic tremor occurs during voluntary movements such as drinking, eating or writing. The intentional tremor can be identified during a finger-to-nose movement if the amplitude of tremor increases when approaching to the target. Postural tremor might also occur in patients with essential tremor when they hold their arms horizontally against gravity. The familial forms of essential tremor show heterogeneity in terms of age at onset, rate of progression of tremor, and tremor distribution (59). Rest tremor, which occurs while a body part is relaxed and supported against gravity, has also been observed in patients with essential tremor (59). It is suggested that motor system abnormalities, such as basal ganglia, might cause the basis of rest tremor in essential tremor (58).

Essential tremor is a slowly progressive disorder. Over the years, tremor might spread from the arms to the other unaffected body regions (58). Moreover, some essential tremor patients might develop Parkinson's disease later in their lives (60).

The worldwide prevalence of ET is 0.9%, increasing up to 4.6% in elderly populations (57). According to a population based study in Turkey, the prevalence of essential tremor is 4.0% among individuals older than 40 (61).

2.2.3 Genetic heterogeneity

ET is genetically heterogeneous (Figure 2.1). The first chromosomal region was mapped at chromosome 3q13 (*ETM1*; OMIM 190300) (62) in a genome-wide study including 16 Icelandic families with 75 affected individuals. ET was segregating in an autosomal dominant pattern. The logarithm of odds (LOD) score was 3.71; however, the highest single-family LOD score was 1.29, below the significant threshold to map a monogenic disorder to a marker. Common variant DRD3 p.S9G (OMIM 126451)

located in the *ETM1* region has been proposed as a risk factor for ET (63) in 23 of 30 French families, but this could not be replicated in other populations (64, 65). Therefore, these results should be taken cautiously.

ETM2 (OMIM 602134) locus was mapped at chromosome 2p22-24 in a large American-Czech family segregating essential tremor in an autosomal dominant pattern with a significant LOD score of 5.92 (66). The same group confirmed the linkage to *ETM2* lous in four independent American families (67). HS1BP3 p.A265G located in the *ETM2* region was found in two families with ET (68). However, subsequent association and linkage studies were not able to identify any association or linkage to this region (69, 70). Thus the causality of *ETM2* remains unknown.

Linkage to chromosome 6p23 (*ETM3*; OMIM 611456) was identified in a genome-wide linkage study including seven American families with 325 affected individuals segregating ET in an autosomal dominant pattern (71). However, sequencing the 15 candidate genes in *ETM3* locus did not identify any pathogenic variants. Two different linkage studies in Italian families with essential tremor excluded any linkage to *ETM3* locus (70, 72). Genome wide association studies reported common intronic variants in *LINGO1* (73) and in *SLC1A2* (74) to be associated with ET, but meta-analysis indicated that it is unlikely that any of these variants are causative (75).

Recently, a nonsense mutation p.Q290X in the RNA-binding protein FUS was identified by whole exome sequencing in a large family with ET (*ETM4*; OMIM 614782) (43). Further screening of ET cases for *FUS* revealed two rare missense variants, (76, 77) suggesting that mutations in *FUS* explain a subset of cases of ET.

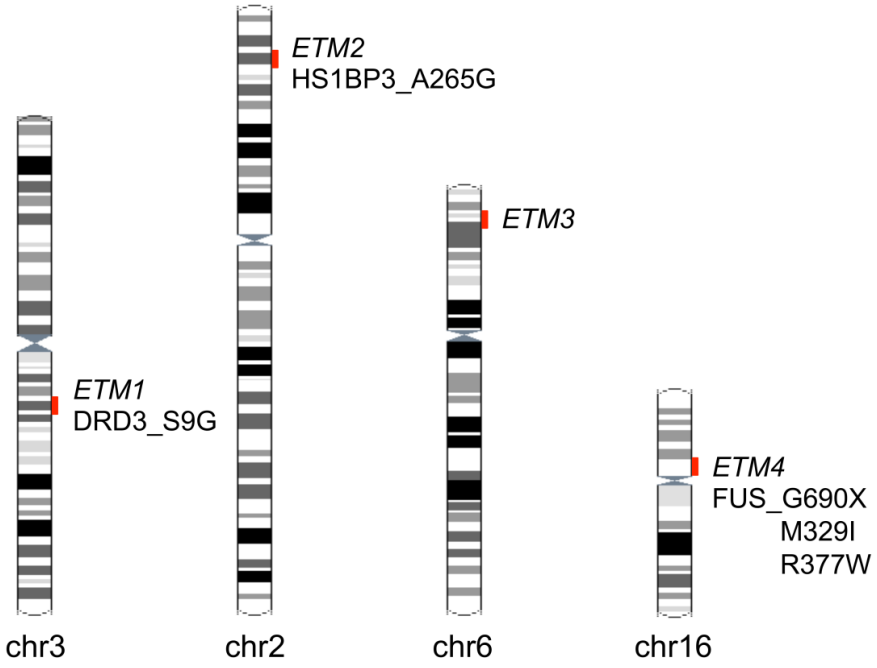

Figure 2.1 Genetic heterogeneity in familial essential tremor. Linkage studies revealed three genomic regions segregating with the condition. No clearly casual mutations have been identified in these regions, except two common variants associated with the disese. Recently a nonsense mutation in the RNA-binding protein FUS was identified by whole exome sequencing.

Another study showed that DNAJC13 p.N855S, which had previously been identified in Parkinson's disease patients, was also present in two unrelated patients with essential tremor (78).

2.2.4 Etiologic and pathologic heterogeneity

Disease etiology refers to the study of initial or primary causes and origin of the disease. The etiological studies showed that both genetic and environmental factors might be operating in combination for the cause of essential tremor (79). Environmental factors play a role in late-onset neurodegenerative disorders like essential tremor, Alzheimer's disease and Parkinson's disease. Case studies show that there are both familial and sporadic forms of essential tremor (58). The causes of these sporadic forms

of essential tremor are not fully known but β-carboline alkaloids and lead are some of the putative environmental factors that have been examined with several case-control studies (80, 81). β-carboline alkaloids are present in human diet, such as in animal proteins, and are found to be elevated in the blood concentration of essential tremor patients than in controls (80). Lead, a neurotoxin which causes cerebellar damage, has also been found to be higher in blood from essential tremor patients than controls (81). However, these studies need to be replicated with additional case-control studies. It is possible to suggest the "presence of a gene-environment or two-hit (increased genetic susceptibility followed by exposure to environmental factors) model" for essential tremor (59).

Disease pathophysiology refers to the study of mechanisms operating within an organism and its changes after the disease occurrence. The pathophysiological basis of essential tremor is relatively unknown. The histopathological examinations of brains of indicated that essential tremor cases can be clustered into two categories: those with cerebellar degenerative changes and those with brainstem Lewy bodies (82). The majority of essential tremor cases have cerebellum with larger amounts of Bergmann glial cells and higher Purkinje cell axons, as well as other microscopic changes in the cerebellum (torpedoes) and have been referred as "cerebellar essential tremor" (Figure 2.2A). Bergmann glial cells are nonspecific pathological response to injury, and torpedoes are swelling of the Purkinje cell axons which results in Purkinje cell loss (83). These findings are not unexpected since the tremor is believed to be mediated by cerebello-thalamo-cortical pathway (58). Moreover, the intentional component of essential tremor further supports an abnormality of cerebellar function (58). A smaller portion of essential tremor cases show Lewy bodies mainly in the locus coeruleus which is distinctive from brainstem

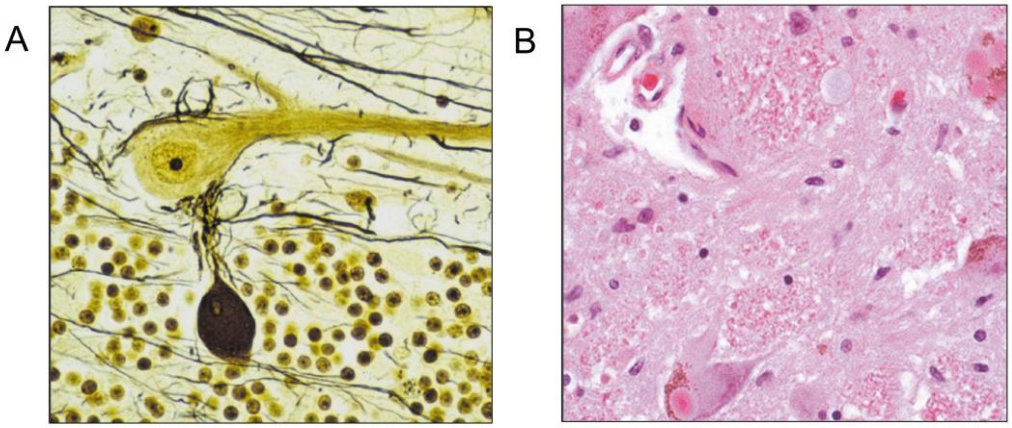

Figure 2.2 Pathological examination of the brains of patients with essential tremor. *(A)* Fusiformal axonal swelling (torpedoe) is observed on a modified silver stain of cerebellar Purkinje cell. Torpedoes are pathological accumulation of phosphorylated neurofilaments. *(B)* Haematoxylin-eosin staining of the locus coeruleus shows multiple Lewy bodies, with as many as four per neuron. (Copyright 2005 Elsevier. From Louis et al. (84) with permission)

Lewy bodies where the patterns are observed prominently in substantia nigra pars compacta (84) (Figure 2.2B). This pattern is different from that of healthy and aged controls in whom Lewy body formation is not observed or very rare (82). It might be suggested that essential tremor patients who develop Parkinson's disease in later ages go on to develop more widespread (such as nigral) Lewy bodies; however, further evaluation is needed.

Therefore, it might be suggested that essential tremor is "a family of diseases rather than a single entity" (85) due to the presence of multiple genes and environmental factors as the cause for the disease, the evidence of variable pathologic patterns, and the broad variety in the clinical features.

2.2.5 Treatments

There is no cure for essential tremor; however, some treatments can ease the symptoms for some patients (86). The cornerstone therapeutic drugs are anticonvulsant primidone and β-adrenoceptor antagonist propranolol (87). Deep brain stimulation (thalamic stimulation) is required to the patients who failed to benefit from the medications (88). Ethanol is also known to release the symptoms of tremor by binding to the $GABA_A$ receptors and facilitate GABAergic neurotransmission; however, there are also serious drawbacks of chronic alcohol usage for treatment (58).

Identification of new causative genes for essential tremor will highlight new biochemical pathways to allow the design of new effective treatments.

2.2.6 Aim of this study

In this study, we examined a 6-generation family (ET-1) segregating ET or ET as a predominant feature of PD. We carried out whole exome sequencing of DNA from three severely affected individuals in the family in order to identify the causative mutation.

2.3 Materials and Methods

2.3.1 Subjects

Family ET-1 is of Turkish origin. The proband was first evaluated at Ankara University Medical School. He and his informative relatives were followed at Ankara University Medical School and Hacettepe University Medical School. This project was approved by the ethics committees of all participating universities and informed consent was obtained from all individuals. Each participant was examined for essential tremor

using the criteria of both the Washington Heights-Inwood Genetic Study of essential tremor (WHIGET) and the Consensus Statement of the Movement Disorder Society (MDS) on Tremor (Table 2.1) (89, 90). Each participant was rated for rest and postural tremors and was asked to perform four different tasks (pouring water, drinking water from a cup, finger-to-nose movement, and drawing spirals) to elicit kinetic tremor. During the examination, severity of tremor was rated during each task (89). Participants were evaluated for features of Parkinson's disease using the diagnostic criteria of the UK Parkinson's Disease Society Brain Bank. Diagnosis of Parkinson's disease required presence of bradykinesia plus at least one of muscular rigidity, resting tremor, or postural instability (91). We collected histories about distribution and severity of tremor and change of these parameters over time. Participants were interviewed about concurrent use of drugs that might cause action or resting tremor and for symptoms of hyperthyroidism, which was ruled out by TSH tests as needed. Clinical assessments were carried out by at least two neurologists without knowledge of participants' genotypes. An additional 25 families with hereditary essential tremor, 59 patients with Parkinson's disease, and 364 healthy controls, ages 20-30 and from the same central Anatolian region as family ET-1, were recruited for genetic analysis from Ankara University Medical School, Hacettepe University Medical School, and Bilkent University. Unaffected individuals showed no signs of disease at the time of examination. Because controls were young adults, they serve as population controls.

2.3.2 DNA isolation

DNAs of all family members were extracted from blood using Nucleospin Blood Kit (Macherey-Nagel) according to manufacturer's protocol.

2.3.3 SNP genotyping, haplotype analysis and identity by descent analysis

DNA samples from five affected subjects (IV:3, IV:4, IV:8, V:8 and VI:5) and one unaffected subject (IV:5) were genotyped using GeneChip mapping 250K NspI SNP arrays and analyzed using GTYPE software (Affymetrix).

Identity by descent (IBD) regions shared by all genotyped affected family members were determined using a pipeline developed by King Lab (Figure B; Table B). SNP genotype data of a tio (unaffected mother (IV:5), affected father (IV:4), and affected daughter (V:8)) was used to determine the hypothetical disease alleles for each informative marker. Each marker was then compared in the additional three affected relatives (IV:3, IV:8, and VI:5). If genotypes of one of the three subjects is opposite homozygous at one marker (e.g. disease allele is A, opposite homozygous is BB) then this marker was called IBD0. Then, IBD0 SNPs were merged into segments to define IBD0 regions. A region was assigned as IBD0 if it contains at least 2 informative IBD0 SNPs in a minimum 500 Kb segment. All IBD0 segments, centromeric and telomeric regions, and sequence gaps were filtered out from the whole genome. Remaining regions with at least 3 Mb in size were considered potential IBD segments.

In order to determine whether essential tremor in family ET-1 is linked to *DNAJC13* or to genomic regions previously reported to be associated with essential tremor (43, 62, 66, 71, 78), haplotype analysis were performed. Haplotypes were generated in a similar method used for IBD analysis to evaluate the possibility of a disease-causing mutation in any of these regions.

Table 2.1 Clinical characteristics of tremor in the affected individuals of family ET-1

Individual	Age at Onset	Age at Exam	Tremor									Archimedes Spiral Test		Bradykinesia		Rigidity		Postural Instability	Hypomimia
			Resting		Postural		Kinetic		Head	Chin	Voice								
			R	L	R	L	R	L				R	L	R	L	R	L		
III:5	50	78	-	+	+	++	+++	+++	Yes	No	Yes	+++	+++	-	-	+	+	-	+
IV:2	40	79	++	+	++	++	++	++	Yes	No	Yes	+++	+++	+	+	-	-	-	-
IV:3	<30	81	-	-	++	+++	++	+++	No	No	No	+++	+++	++	++	+++	++	+++	++
IV:4	<30	89	-	-	++	+++	++	+++	No	No	No	+++	+++	++	++	+++	++	+++	++
IV:8	40	81	++	+	++	+++	++	+++	No	Yes	No	++	+	+++	++	+++	++	+	+
IV:13	59	67	-	-	+	++	+	++	No	No	No	+	+	-	-	+	-	-	-
IV:18	10	45	+	+	+++	+++	+++	+++	No	Yes	Yes	+++	+++	-	-	-	-	-	+
V:3	48	56	-	-	+	+	+	+	No	No	No	+	+	-	-	+	-	-	-
V:4	30	61	-	+	+	+	+	+	No	No	No	+	+	-	+	+	+	-	-
V:6	50	60	-	-	+	+	-	-	No	No	No	-	-	-	-	+	-	-	-
V:7	<55	59	-	-	++	+	+	++	No	No	No	+	+	-	-	-	-	-	-
V:8	12	50	+	+	+++	+++	+++	+++	Yes	No	No	+++	+++	-	+	-	+	-	+
V:9	15	56	+	++	++	+++	+	++	No	No	No	+++	++	++	+	+	+	-	+
VI:5	10	25	-	+	+++	++	++	++	No	No	No	++	++	-	-	-	-	-	+

Abbreviations: For tremor +: low amplitude or barely perceivable tremor, ++: moderate amplitude tremor (1-2 cm), +++: large amplitude, severe tremor (>2 cm).(89) For Archimedes spiral test, bradykinesia, rigidity, postural instability and hypomimia +: mild, ++: moderate, +++: severe. R: right hand, L: left hand. None of the individuals had intentional tremor. Subject IV:9 was deceased and is not included in the table. Subject VI:5 was assessed based on his clinical history and is not included in the table.

2.3.4 Library construction and whole exome sequencing

Three severely affected individuals (IV:3, IV:4 and VI:5) were selected for whole exome sequencing and their high quality DNA's were isolated (Table 2.2).

Table 2.2 Concentrations and qualities of DNA isolated for whole exome sequencing from ET-1 family

Sample ID	Concentration (ng/µl)	$A_{260/280}$	$A_{260/230}$
IV:3	215.7	1.9	2.0
IV:4	252.1	1.9	2.2
VI:5	131.5	1.8	1.7

Library construction for whole exome sequencing was carried out according to manufacturer's protocol (Illumina TruSeq DNA Sample Preparation Guide). For whole exome sequencing, DNA samples were diluted in suspension buffer to a final amount of 2.5 µg in 55 µl. DNA was sheared into 200-300 bp for 120 s in sonicator at 6ºC (Covaris) in order to generate dsDNA fragments with 3' or 5' overhangs. The sheared DNA was run on a gel for quality check; the final amount of the sheared DNA should be 1 µg. End repair was performed in order to convert the overhangs resulting from fragmentation into blunt ends by incubating the fragmented DNA with End Repair Mix for 30 min at 30ºC. The fragmented DNA was cleaned up using AMPure XP Beads. 160 µl AMPure XP Beads were added to each sample and this mixture was placed to magnetic stand. The DNA samples bound to beads were washed with 80% ethanol and dried. DNA was resuspended in Resuspension Buffer. This was followed by A-tailing by incubating the DNA fragments with A-tailing mix for 30 min at 30ºC. This reaction allows single 'A' nucleotides to be added to the 3' ends of the blunt fragments to prevent them from ligating to one another during adapter ligation. Multiple paired-end

indexing adapters with corresponding T nucleotides on their 3' ends were ligated to the complementary dsDNA overhangs by incubating at 30ºC for 10 min. The ligation reaction was inactivated by the addition of Stop Ligation Buffer. This step provided DNA fragments for hybridization onto a flow cell. The DNA fragments were cleaned up with AMPure XP Magnetic Beads. After this, DNA fragments with adapters on both ends are amplified with PCR. DNA quality was checked at this step by running on Agilent Technologies 2220 TapeStation instrument. Exomes were captured by SeqCap EZ Exome v2 (Roche) and hybridized to biotinylated capture probes at 47 ºC for 72h. The post-captured exomes were amplified with LM-PCR. The amplified captured exomes were cleaned up using Qiagen QIAquick PCR Purification Kit, and the final concentration and quality were calculated by running on Agilent D1K ScreenTape and analyzing with Agilent Technologies 2220 TapeStation software (Figure 2.3). Libraries were sequenced on an Illumina HiSeq2500.

2.3.5 Bioinformatics

Paired-end sequence reads were aligned to the human reference genome (hg19) using Burrows-Wheeler Aligner (BWA, v0.6.1-r104) (92). Removal of PCR duplicates, sorting and indexing were done using SAMtools v0.1.18 (93). Indel realignments and base quality score recalibration (BQSR) were done with Genome Analysis Tool Kit (GATK, v3.0-0-g6bad1c6) using recommended parameters (94). Genotypes were called and filtered using GATK Unified Genotyper and Variant Filtration tools. Variants were annotated using our in-house pipeline. Common SNPs and artifacts were excluded using dbSNP v138, the NHLBI Exome Sequencing Project, the 1000 Genomes Project, and 700 exomes previously sequenced in our lab. Variants were defined as potentially damaging if they led to premature stop codon or were mutations with scores on *in silico*

prediction tools SIFT P≤ 0.05, PolyPhen2 score ≥ 0.8, and MutationAssessor score ≥ 1.95 (Table S2) (46-48).

2.3.6 Sanger sequencing

Genotypes for candidate variants for 24 informative relatives of family ET-1 were determined by capillary sequencing (ABI 3130xl Genetic Analyzer). All coding regions, potential regulatory regions and miRNA binding sites of *HTRA2* were sequenced in probands from 25 unrelated families with multiple relatives with essential tremor. Primers were designed using Primer3 (Table C1) (95). Products were analyzed via gel electrophoresis and Sanger sequenced. Sanger traces were analyzed with CLCBio Main Workbench software package (CLCBio Inc.). Family ET-1 was genotyped with FAM- and HEX- labeled primers for polymorphic markers on chromosome 2 flanking *HTRA2* (ABI 3130xl Genetic Analyzer). Data was analyzed with GeneMapper v4.0 software package (Applied Biosystems).

2.3.7 Population screening

TaqMan genotyping assay (Life Technologies) was used for screening HTRA2 p.G399S in Parkinson's disease patients and controls. Custom TaqMAn SNP genotyping assay was designed to amplify and detect both alleleles with probes labeled with FAM or VIC dyes. One homozygous, one heterozygous, two no template controls and 364 Turkish control DNA samples (10 ng each) were added into each well of 384 optical plate. TaqMan Universal PCR Master Mix (No AmpErase UNG) (2X) and SNP Genotyping Assay (20X) were mixed into the required volume and added into the each well of 384-DNA reaction plate. The reaction was performed on ABI 7900HT Fast

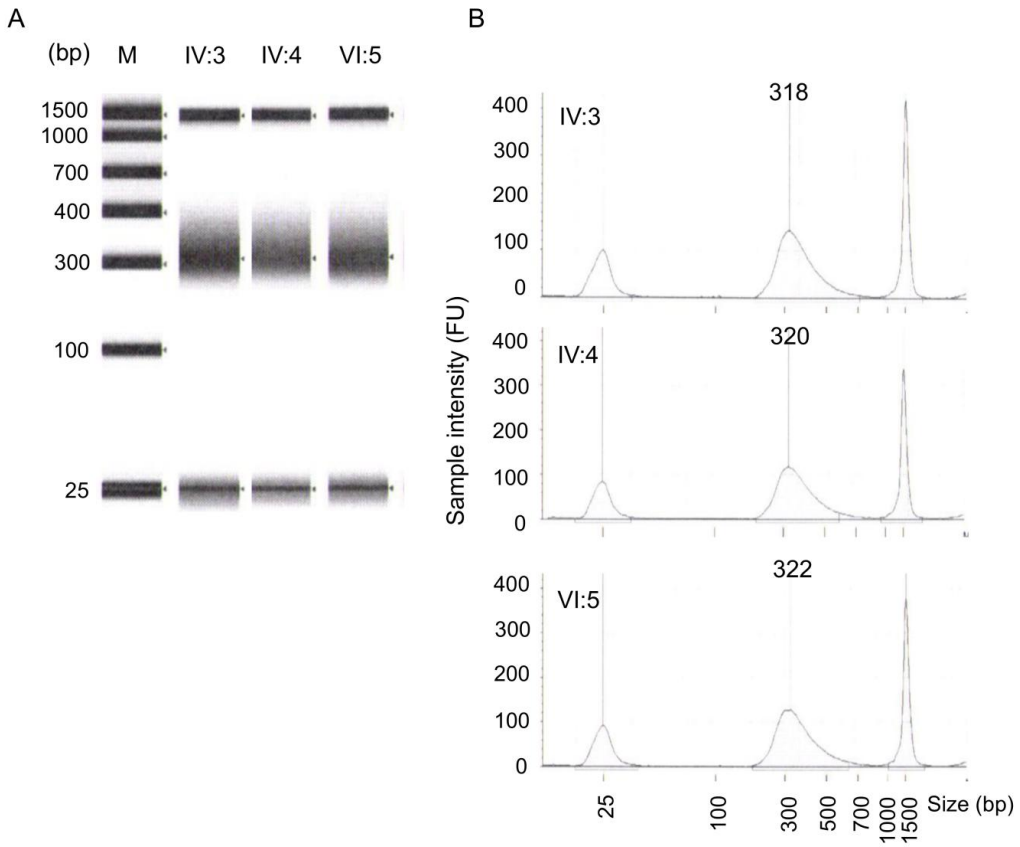

Figure 2.3 Quality control of post captured amplified whole exome library. *(A)* Gel image of post-captured DNA library. 1500 bp and 25 bp are intrinsic upper and lower markers, respectively. (B) Histogram representation of the results from panel *A*. bp: basepair, M: marker.

Real-Time PCR System with optimized conditions. After PCR amplification, an allelic discrimination plate read was set and performed, and the automatic allele calls were converted into genotypes (Figure D).

2.3.8 Linkage analysis

A LOD score for linkage of *HTRA2* to essential tremor in the ET-1 family was calculated using LINKAGE v6.0 (96) under an autosomal dominant mode of inheritance with penetrance for homozygous or heterozygous genotypes of 1.0 at age 40 and older and 0.6 before age 40, no phenocopies, and a mutant allele frequency of 0.01 in the general population.

2.3.9 Statistical analysis

Statistical significance was evaluated by one-way analysis of variance (ANOVA), or analysis of covariance (ANCOVA), as appropriate.

2.4 Results

2.4.1 Clinical features of the ET-1 family

The family is from central Anatolia, where consanguineous and endogamous marriages are common practice. Ancestors of the extended family have lived in the same area for more than 400 years. Essential tremor is known to have segregated in the family for generations. Twenty-four individuals from the family were clinically assessed (Figure 2.4). Sixteen family members showed varying degrees of tremor (Table 2.1; Figure 2.5), eleven of whom were diagnosed with ET and five of whom (IV:2, IV:3, IV:4, IV:8 and V:9) were diagnosed with ET co-existing with PD.

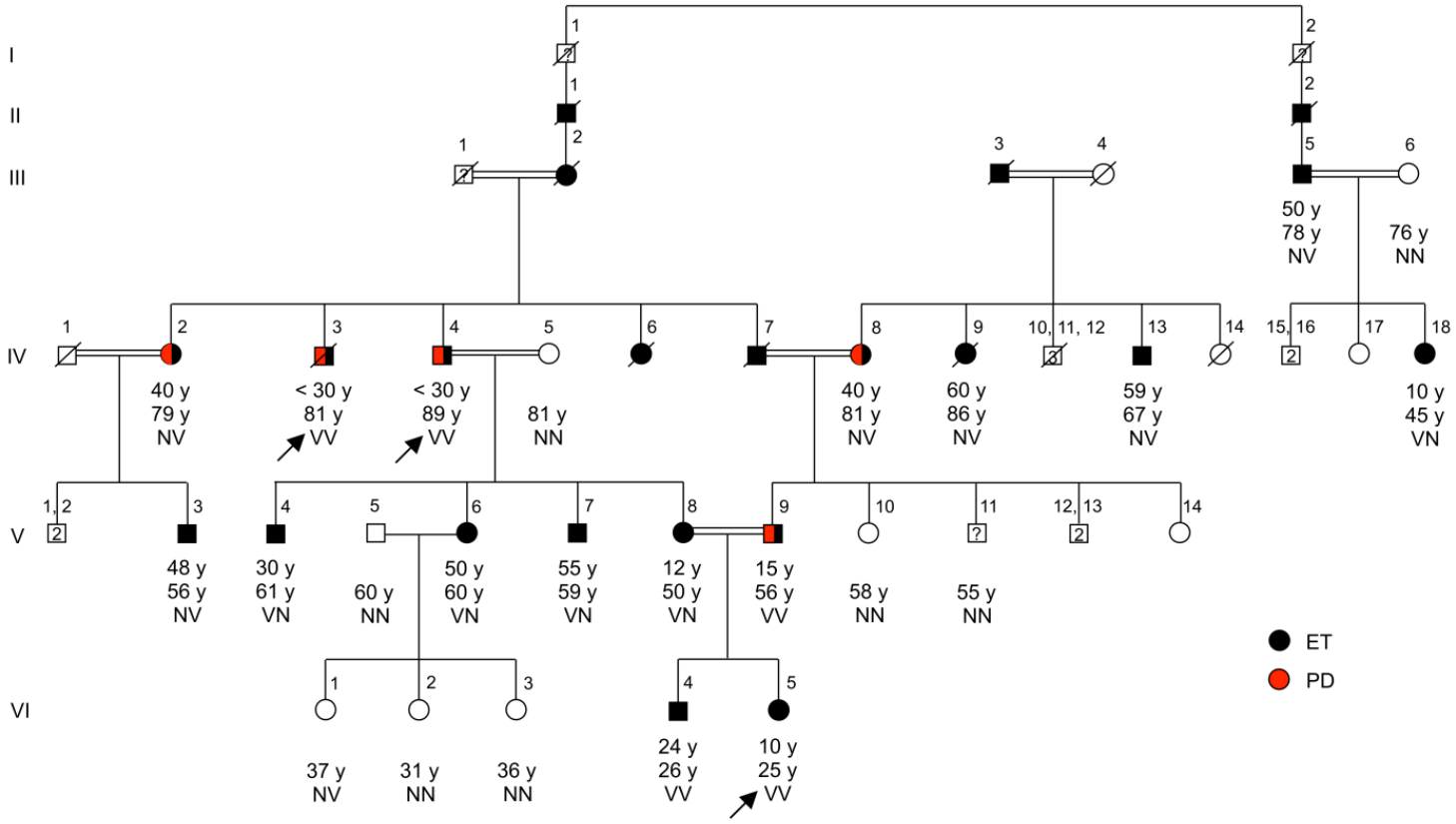

Figure 2.4 Pedigree of family ET-1 segregating essential tremor, with genotypes at HTRA2 G399S. Pedigree of family ET-1.Arrow indicates the proband. Individuals with ET are shown with filled black symbols, and with PD are shown with filled red symbols. Age at onset for affected individuals, and current ages and genotypes are indicated under the symbols. Individuals who underwent exome sequencing (IV:3, IV:4, VI:5) are represented with asterisks. V indicates the variant allele (Ser) at HTRA2 G399S; N indicates the wildtype allele (Gly). V:11: declined clinical examination.

Diagnoses of ET co-existing with PD were made as follows. IV:3 (age 81), IV:4 (age 89), and V:9 (age 56) developed mild to moderate postural and action tremor before age 30 and bradykinesia, rigidity, resting tremor, and postural instability with increasing age. IV:2 (age 79) developed postural and action tremor at middle age and subsequently bradykinesia and resting tremor. IV:8 (age 81) developed postural and action tremor at middle age and subsequently resting tremor, bradykinesia, rigidity, and postural instability. For all five of these patients, all the indicated signs were apparent upon physical examination for this study. Subject V:8, whose clinical features were particularly severe, was diagnosed with ET at age 12 and with Hashimoto's thyroiditis at age 49. Subject IV:13 was using propranolol at the time of examination. Subjects V:3, V:4, V:6 and V:7 had mild tremor of at least 4 years' duration. VI:4 has mild tremor with an age at onset of 24 according to his medical history, but his clinical scores could not be obtained by a face-to-face physical exam. Subject IV:9 provided a DNA sample but died before clinical evaluation; according to her clinical history she had mild hand and head tremor. Subject V:11 agreed to provide a DNA sample but declined subsequent clinical evaluation. Ages of onset of tremor ranged from ~10 years to 60 years. Some family members with tremor were not able to determine the exact age of onset as their tremor developed over many years. ET appeared to be inherited as an autosomal dominant trait spanning six generations.

2.4.2 Gene discovery

Results of exome sequencing of three severely affected relatives, IV:3, IV:4 and VI:5, are summarized in Table 2.3. The proportion of targeted genomic regions covered at least 8-fold was 95.1% for IV:3, 95.4% for IV:4, 94.6% for VI:5, and 93.6% jointly (Figure E1). Given that the kindred includes multiple consanguineous marriages, we

first considered the possibility of recessive inheritance of essential tremor as the result of homozygosity for a critical mutation that was identical by descent from a common ancestor. To evaluate this possibility, we identified homozygous genomic regions greater than 1 MB shared by the three affected relatives. There were three such regions, on chromosomes 2p13.1-p12 14q32.13, and 22q11 (Table 2.4). We then identified, in each of these regions, all variants predicted to be damaging (see Methods). The only potentially damaging variant was HTRA2 p.G399S (c.1195G>A, NM_013247) at chr2:74,759,825 G>A (rs72470545). HTRA2 p.G399S was predicted to be damaging by bioinformatics prediction tools Polyphen2 (score 0.986), SIFT (P = 0.02) and MutationAssessor (score 2.39).

We next genotyped HTRA2 p.G399S in all family members. Of the 16 individuals with essential tremor in the family, five were homozygous and 11 were heterozygous for the variant (Figure 2.4). The occurrence of both heterozygotes and homozygotes for the mutation among affected relatives precluded recessive inheritance via identity by descent. On the other hand, genotypes of the family were consistent with dominant inheritance of essential tremor due to this allele, possibly with a dosage effect.

In order to evaluate the kindred for the possibility of dominant inheritance of any mutation in the genome, we next identified all potentially damaging variants, whether heterozygous or homozygous, shared by the three affected relatives with exome sequence. Including HTRA2 p.G399S, there were 13 such variants, all missenses (Table 2.5). We genotyped all 13 variants in all family members. The only variant segregating with essential tremor in the family was the *HTRA2* allele (Figure 2.4; Table 2.5; Figure 2.6). The LOD score for linkage of HTRA2 p.G399S under an autosomal dominant model of inheritance was 5.27.

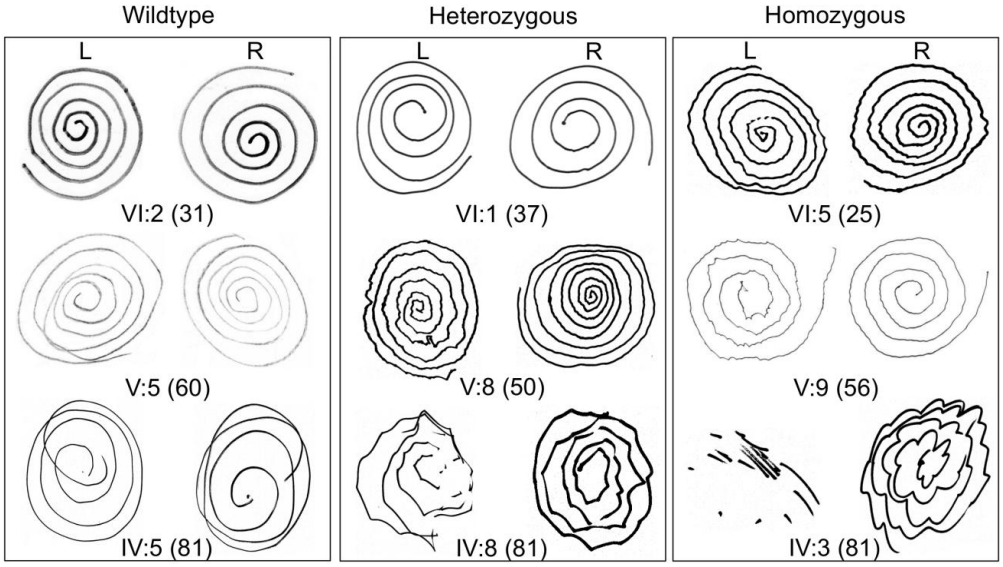

Figure 2.5 Archimedes spiral tests of individuals of various ages and genotypes at HTRA2 p.G399S. For all individuals R: dominant right hand, L: non-dominant left hand.

We next considered the possibility that affected family members heterozygous for HTRA2 p.G399S might carry a second damaging allele of *HTRA2* on their other copy of chromosome 2. In order to evaluate this possibility, two tests were carried out. First, *HTRA2* was fully sequenced in all family members. No rare variants other than p.G399S were identified. Second, to test the possibility of a critical non-coding regulatory mutation of *HTRA2* shared by affected family members, extended (3.7 MB) haplotypes flanking *HTRA2* were determined for all relatives using informative polymorphic markers (Figure 2.7). Subjects heterozygous for HTRA2 p.G399S did not share a second haplotype. We concluded that the possibility of a second pathogenic *HTRA2* allele segregating in this family is extremely unlikely. We also evaluated and excluded the possibility of linkage of essential tremor to a mutation in any of the genomic regions previously reported to be associated with the disorder (Figure F).

Several lines of evidence suggested that the number of copies of HTRA2 p.G399S influenced severity of the phenotype. First, results of the Archimedes spiral test (89) suggested that severity of action tremor was influenced by both genotype and age (Figure 2.5). Second, homozygosity versus heterozygosity at HTRA2 p.G399S was significantly associated with age at onset of tremor ($F = 28.99$ [2, 24 df], $P < 0.0001$; Figure 2.8A). Mean ages at onset of tremor were 21.4 years and 41.3 years for subjects homozygous and heterozygous for the mutation, respectively. Third, homozygosity versus heterozygosity at HTRA2 p.G399S was associated with severity of both postural tremor ($F = 18.68$ [2, 17 df]), $P < 0.0001$; Figure 2.8B) and kinetic tremor ($F = 9.24$ [2, 17 df]), $P = 0.0019$; Figure 2.8C).

Table 2.3 Results of whole exome sequencing of DNA from three severely affected relatives from ET-1

	IV:3	IV:4	VI:5
Total number of reads	82,905,251	88,364,432	83,032,868
% of mapped reads	98.1%	98.3%	97.6%
% of targeted bases covered ≥ 8X	95.1%	95.4%	94.6%
Joint coverage at ≥ 8X[a]		93.6%	
Average coverage	90X	101X	83X
All coding variants	18,729	18,839	18,198
Shared by all 3 exomes		11,639	
Rare variants[b]		129	
Predicted damaging to protein function[c]		13	
Co-segregation with ET in the family		1 (HTRA2 G399S)	

Abbreviations.

[a]Joint coverage was defined as fold-coverage for the least well-covered individual of the three samples.

[b]Filtered by dbSNP138 common SNPs and MAF ≥ 0.005 in EVS or 1000G variants, and proportion of variant reads ≥ 0.35. Both heterozygous and homozygous variants were included.

[c]Truncating mutations, splice site mutations predicted to lead to altered transcripts, and missense variants with all of Polyphen2 score ≥ 0.8, SIFT score ≤ 0.05 and MutationAssessor score ≥ 1.95 were defined as damaging. Both heterozygous and homozygous variants were included.

Table 2.4 Homozygous regions shared by three affected relatives

Chr	Start	End	Size (bp)	All SNPs	Rare potentially functional SNVs and indels[†]
2	73,518,867	75,115,108	1,596,241	48	1
14	94,912,799	96,157,331	1,244,532	41	0
22	16,953,727	18,650,682	1,696,955	77	0

Homozygosity mapping from whole exome data was performed using PLINK V1.07 with 200 kb minimum segment size (1). In the shared homozygous regions, 98% of all coding bases were sequenced at least 4X in at least one subject with average coverage of 76X.

[†]Alleles predicted damaging to protein function were of the following classes: truncating mutations; splice site mutations predicted to lead to altered transcripts; whole gene deletions; and missense variants with Polyphen-2 score $\geq$ 0.8, SIFT score $\leq$ 0.05 and Mutation Assessor score $\geq$ 1.95.

Table 2.5 Damaging coding sequence variants shared by exome sequences of three severely affected relatives of family ET-1

Chr	Start	Ref	Var	Gene	Effect	Genotype	PPH2	SIFT	MA	Gerp	EVS	1000G	Subjects with tremor		
													NN	NV	VV
2	55,194,157	C	T	EML6	R1839C	Het	0.998	0.022	2.25	6.17	-	0.0009	4	10	2
2	**74,759,825**	**G**	**A**	**HTRA2**	**G399S**	**Homoz**	**0.986**	**0.020**	**2.39**	**3.99**	**0.0034**	**0.0023**	**0**	**11**	**5**
3	13,359,234	G	A	NUP210	R1871C	Het	0.999	0.021	2.07	4.58	-	-	11	5	0
4	1,343,416	T	C	UVSSA	L68P	Het	1	0.001	3.02	4.98	-	-	10	6	0
6	13,306,697	A	G	TBC1D7	L243S	Het	1	0	2.67	5.87	-	-	9	7	0
9	104,190,765	T	C	ALDOB	E122G	Het	0.997	0	3.36	5.87	0.0001	-	9	7	0
9	135,203,279	C	A	SETX	V1236F	Het	0.868	0.003	1.95	4.82	-	-	5	11	0
12	52,284,475	C	T	ANKRD33	R124W	Het	1	0.001	2.28	-	0.0005	-	11	5	0
15	41,797,248	C	T	LTK	R647Q	Het	1	0	2.50	3.79	0.0003	-	11	5	0
15	58,004,256	G	A	GCOM1	R675Q	Het	0.999	0.036	1.99	2.79	-	-	6	10	0
15	90,328,681	G	A	ANPEP	R935W	Het	1	0.013	3.12	5.31	0.0009	0.0005	11	5	0
17	31,098,168	T	C	MYO1D	Y230C	Het	0.998	0	3.48	5.82	0.0008	-	10	6	0
19	48,565,262	G	A	PLA2G4C	P417L	Het	0.819	0.013	2.48	2.79	-	-	9	7	0

Abbreviations: Chr: chromosome, Ref: reference base, Var: variant base, PPH2: Polyphen2, MA: MutationAssessor, EVS: Exome variant server, 1000G: 1000 genomes project, V: variant allele, N: wildtype allele.

Of 59 other Turkish individuals with Parkinson's disease, none carries HTRA2 p.G399S. In 25 other Turkish families, each including multiple relatives with essential tremor, complete sequencing of *HTRA2* did not reveal any damaging mutations. Of 364 unrelated Turkish controls, two were heterozygous and none were homozygous for HTRA2 p.G399S, yielding an allele frequency of 0.0027, approximately the same as reported in a population of mixed European ancestry (Table 2.5). Of the two controls heterozygous for the allele, one was anonymous and the other had given permission to be re-contacted. The re-contacted control is presently 27 years old. Upon neurological exam, she had no signs of essential tremor.

2.5 Discussion

Whole exome sequencing of three severely affected relatives of family ET-1, with no prior hypothesis about a causal gene, revealed HTRA2 p.G399S as the only potentially damaging allele co-segregating with essential tremor in the extended kindred. Homozygosity *versus* heterozygosity for this allele was associated with earlier age at onset and increased severity of essential tremor. Homozygotes for the mutation expressed a more severe phenotype, including signs of Parkinson's disease at middle age, which suggested a dosage effect for this allele. Among heterozygotes, variability in age at onset of tremor could be influenced by genetic or environmental modifiers or stochastic effects (59).

HTRA2 encodes a serine protease of 458 amino acids that localizes to the inter-membrane space of mitochondria (97). Upon an apoptotic stimulus, the HTRA2 protein is released from the mitochondria into the cytosol and binds to inhibitor-of-apoptosis (IAP) proteins in order to initiate apoptosis (97). HTRA2 proteolytic activity also triggers caspase-independent cell death (98).

Several lines of evidence suggest involvement of HTRA2 in Parkinson's disease. HtrA2 p.S275C leads to loss of protease activity and to a motor neuron degeneration phenotype with ataxia, repetitive movements, and akinesis in *mnd2* mice (99). In addition, *HtrA2* knockout mice show parkinsonian features due to the loss of neurons in striatum (100). Based on these observations, Strauss and co-workers sequenced *HTRA2* in a series of German Parkinson's disease patients and controls, and identified HTRA2 p.G399S (the mutation of family ET-1) and HTRA2 p.A141S as associated with the disorder (Figure 2.9A) (101). They also identified HTRA2 in Lewy bodies of Parkinson's disease patients (101). Their study suggested that HTRA2 p.G399S leads to loss-of-function, because both missense mutations (HTRA2 p.G399S and HTRA2 p.A141S) led to mitochondrial dysfunction, altered mitochondrial morphology, and decreased protease activity, and HTRA2 p.G399S resulted in increased sensitivity to toxicity (101). A recent study of transgenic mice also suggested that HtrA2 p.G399S is a loss-of-function allele. In these experiments, mice over-expressing a wild-type allele of HtrA2 showed significant motor impairments, whereas mice over-expressing HtrA2 p.G399S showed no difference compared to non-transgenic mice. (102).

In order to experimentally evaluate a dosage effect for this allele, it would be ideal to compare animal models heterozygous and homozygous for HtrA2 p.G399S. These animals have not been developed, but a clue to a possible dosage effect appears from the comparison of wildtype, $HtrA2^{+/-}$, and $HtrA2^{-/-}$ mice (100). Compared to wild-type mice, $HtrA2^{+/-}$ mice appear to show a subtle, though not statistically significant, decrease in performance on sensory-motor tests (100). Increasing the number of $HtrA2^{+/-}$ mice in these experiments and extending their evaluation over a wider age range would provide valuable information on the possibility of a dosage effect for *HTRA2* loss-of-function mutations.

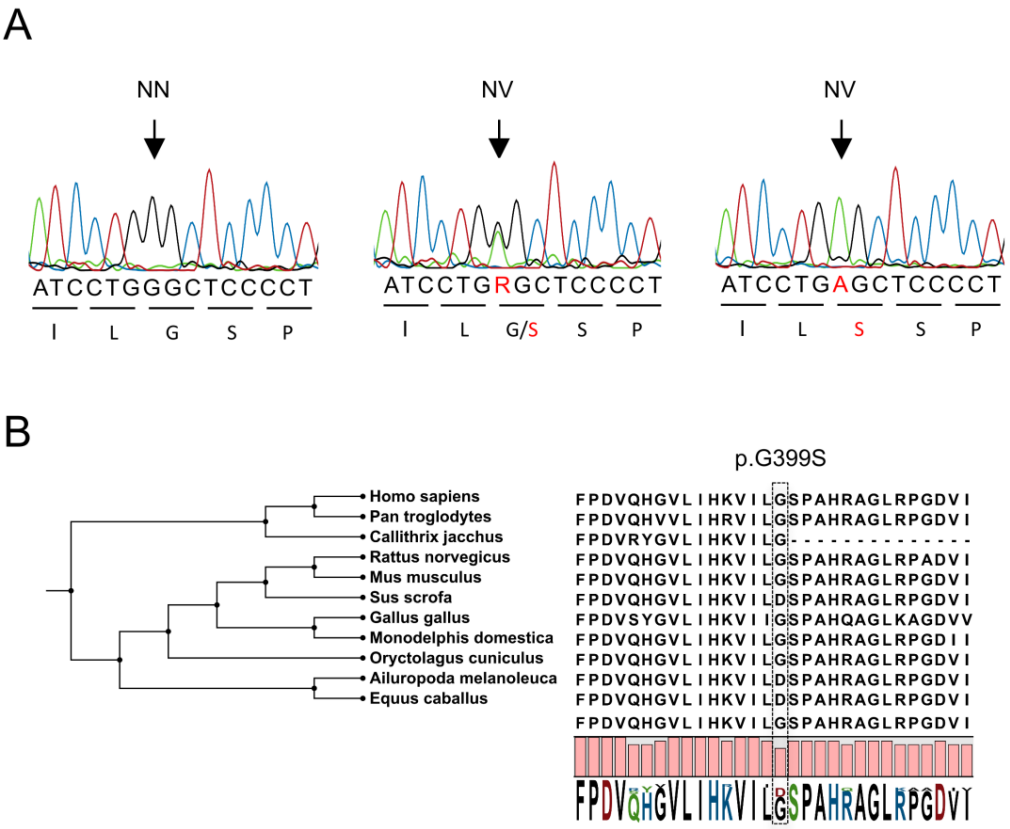

Figure 2.6 Sanger traces of HTRA2 p.G399S in ET-1 family and its conservation. Sanger traces of individuals in ET-1 family and sequence homology of HTRA2 protein. *(A)* Sanger traces of an unaffected wildtype individual, an unaffected heterozygous parent, and an affected homozygous patient. The arrows show the mutation site. *(B)* Sequence homology of HTRA2 protein among various species.

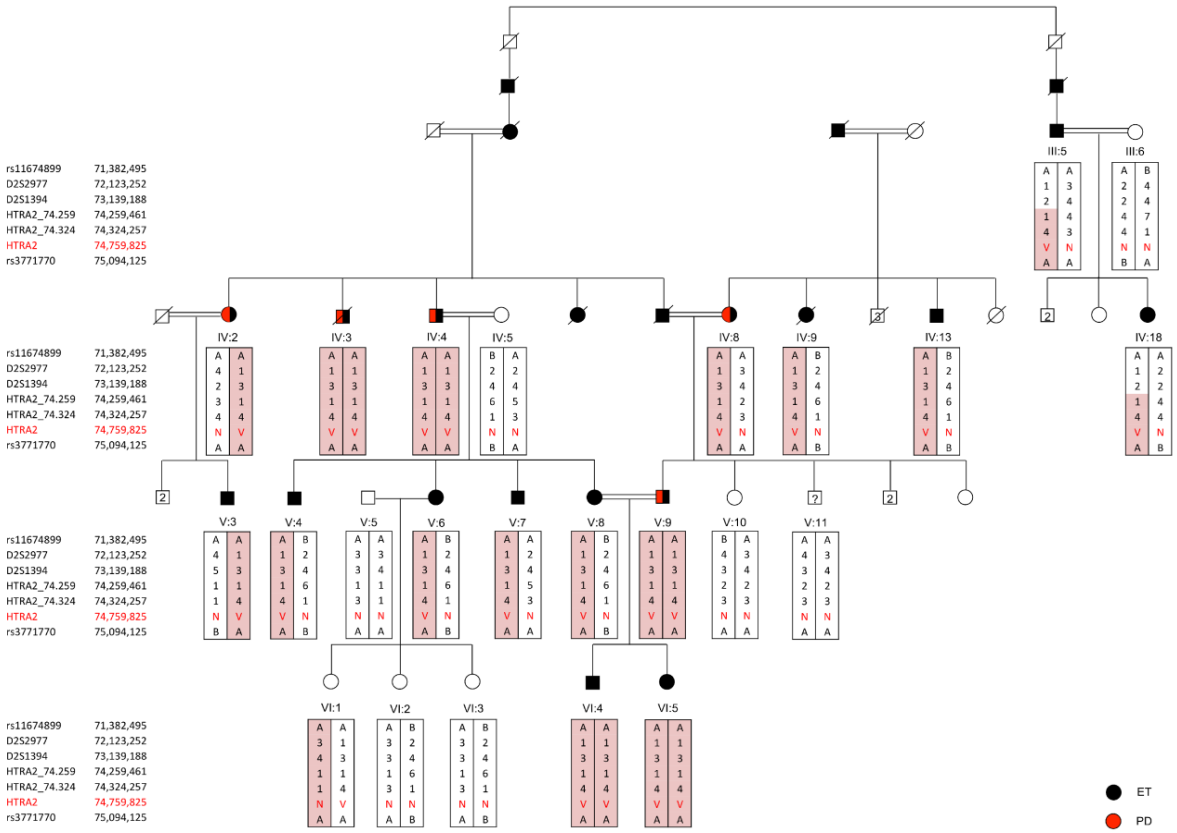

Figure 2.7 Haplotype structure at the *HTRA2* locus for the ET-1 family. All participating family members were genotyped for six informative markers spanning 3.7 MB flanking *HTRA2* and haplotypes constructed by direct pedigree analysis. The 12 subjects heterozygous for HTRA2 p.G399S harbor 9 different haplotypes for their alternate allele, providing strong evidence against a second pathogenic mutation closely linked to *HTRA2*

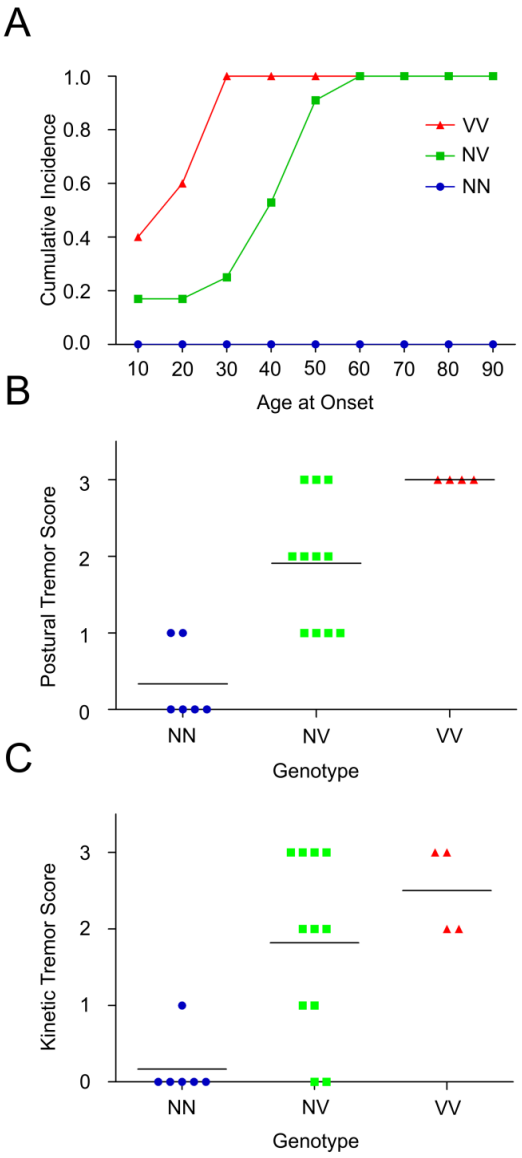

Figure 2.8 Relationship between *HTRA2* genotype and age at onset and severity of tremors of essential tremor. V indicates the variant allele serine and N indicates the wildtype allele glycine at HTRA2 p.G399S. Subjects heterozygous for the variant allele are indicated NV and those homozygous for the variant allele are indicated VV. (*A*) Essential tremor age at onset varies significantly by genotype, $P < 0.0001$. (*B*) Severity of postural tremor of homozygous and heterozygous subjects differs significantly; analysis of covariance by genotype with age at exam as covariate yields $F = 18.68$, (2, 17 df), $P < 0.0001$. (*C*) Severity of kinetic tremor of homozygous and heterozygous subjects differs significantly; analysis of covariance by genotype with age at exam as covariate yields $F = 9.24$, (2, 17 df), $P = 0.0019$.

Three other rare missense alleles, HTRA2 p.R36W, p.P143A, and p.R404W, were subsequently identified in patients with Parkinson's disease (Figure 2.9A) (103-105). HTRA2 p.A141S and p.P143A lie in close proximity to serine at residue 142, which is phosphorylated upon the activity of p38, dependent on PTEN-induced-putative-kinase (PINK1) (Figure 2.9B) (106, 107). HTRA2 p.G399S and p.R404W lie in close proximity to serine at residue 400, which is phosphorylated upon activation of cyclin-dependent kinase-5 (CDK5) (106, 107). In transgenic mice expressing HtrA2 p.G399S in cortex, phosphorylation at residue 400 was significantly reduced (107). Phosphorylation of HTRA2 at both sites is important for cellular stress response (107). Furthermore, PINK1 and CDK5 kinases are both known to be associated with Parkinson's disease (108, 109). However, despite the biological plausibility of a role for *HTRA2* in Parkinson's disease, subsequent epidemiologic studies did not detect higher frequencies of any of the rare missense alleles of *HTRA2* among Parkinson's disease patients than among controls (103-105, 110).

The role of *HTRA2* in essential tremor may resolve this paradox. The individuals in family ET-1 who developed features of Parkinson's disease exhibited these symptoms decades after onset of essential tremor. These included all three individuals homozygous for HTRA2 p.G399S and older than age 55, and two individuals heterozygous for HTRA2 p.G399S and older than age 78. These observations suggest that *HTRA2* has a causal role in essential tremor and in the subset of essential tremor followed by Parkinson's disease. Among essential tremor patients generally, the incidence of Parkinson's disease is increased 4-to-5-fold, and essential tremor and Parkinson's disease have previously been observed in the same families (111-113). We speculate that case-control studies of that subset of Parkinson's disease preceded by essential tremor would reveal associations with functional missense alleles of *HTRA2.*

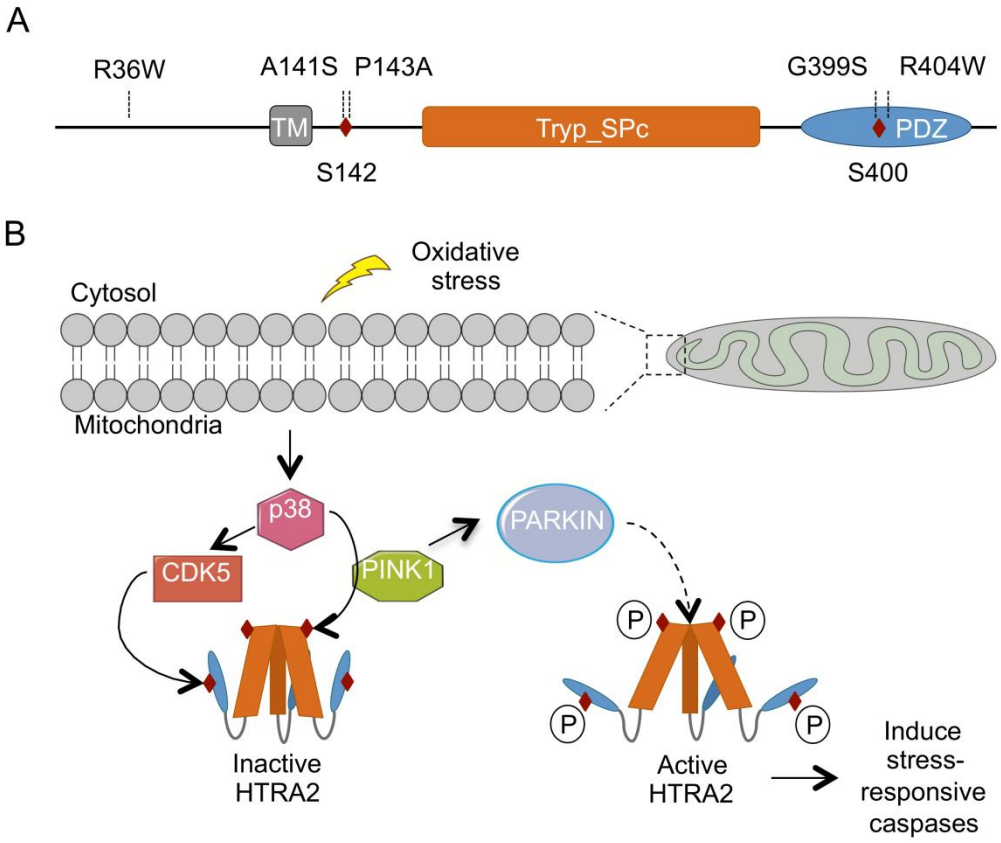

Figure 2.9 Schematic representation of HTRA2 protein and its activation against mitochondrial stress. (A) Localization of the previously identified mutations in HTRA2. Full length HTRA2 consists of a putative transmembrane (TM) domain (aa 105-124), a conserved catalytic trypsin-like serine protease (Tryp_SPc) domain (aa 178-342), and a C-terminal PDZ domain (aa 363-445). (B) Oxidative stress results in the activation of p38 stress kinase pathway which phosphorylates HTRA2 to increase its proteolytic activity. p38 phosphorylates S142 in a PINK1-dependent manner; while S400 is phosphorylated by CDK5. Active HTRA2 induces stress-responsive caspases. PINK1 also recruits Parkin, E3 ubiquitin protein ligase, from cytosol to mitochondria to induce mitophagy. Red diamonds represent two potential phosphorylation sites at S142 and S400. P: phosphorylation.

Although essential tremor is one of the most common inherited neurologic disorders, identifying the responsible underlying genes has been challenging. Complexities of essential tremor include genetic heterogeneity, age-dependent penetrance, and variable expressivity, leading to difficulties both in differential diagnosis and in genetic analysis (59). Our results suggest that mutation of *HTRA2* can be responsible for essential tremor in some families and that parkinsonian features may develop in these patients, after age 70 in heterozygotes and in middle age in homozygotes. These observations reveal one cause of essential tremor and may illuminate some of the shared features of essential tremor and Parkinson's disease phenotypes.

Chapter 3

3 A missense mutation in SPG20 cause Troyer Syndrome

The majority of the work described here is in preparation for publication.

3.1 Outline

Hereditary spastic paraplegias (HSP) are neurological disorders with cardinal features of lower extremity spasticity and weakness. Troyer syndrome is an autosomal recessive complicated HSP caused by loss of function mutations in *SPG20* gene. Here we report a consanguineous Turkish family with Troyer syndrome, where we identified a homozygous missense mutation p.G580R in *SPG20* using whole exome sequencing of two affected siblings. This study indicates a phenotype-genotype relationship that although loss-of-function mutations cause Troyer syndrome, a similar phenotype without skeletal abnormalities was observed with a missense mutation in the same gene.

3.2 Introduction to Hereditary Spastic Paraplegias

3.2.1 Clinical and genetic features of Hereditary Spastic Paraplegias

Hereditary spastic paraplegias (HSP) are characterized by progressive lower weakness and spasticity with a prevalence between 3 to 10 per 100,000 individuals (114). In addition to lower limb spasticity and weakness, which is observed in pure HSPs, other neurologic and non-neurologic abnormalities are observed in patients with

complicated HSPs; for example, intellectual disability, ataxia, peripheral neuropathy, distal muscle wasting, dementia, or optic neuropathy (115). The disease course shows extreme variability, including age at onset, presence of other symptoms, and the degree of weakness in the extremities.

This variability in clinical presentation comes from the variability in the genetics of the disease. There are more than 70 genetic types of HSPs segregating with autosomal dominant, autosomal recessive, X-linked or mitochondrial inheritance patterns (115). HSP genetic loci are represented as *SPG* for spastic paraplegia. Among these disorders, *SPG3A*, *SPG4*, and *SPG11* are relatively well understood due to a large number of patient sizes. The majority of HSPs have not been characterized in many families; therefore, their clinical features are not well known.

The clinical features of HSPs overlap with many disorders. The differential diagnosis of HSPs includes abnormalities in the brain and spinal cord, leukodystrophy, some of the infectious disorders, other neurodegenerative disorders with motor neuron impairment, and environmental toxins (115). The symptoms of HSPs begin in early childhood and they remain relatively non-progressive even over several decades (116). The difficulties in diagnosis occur when there is no family history of similar characteristics, the durations of symptoms are too short to exclude other disorders, there are other neurologic abnormalities, and the genetic testing is not complete. Identification of a potentially pathogenic gene which has previously known to be causing HSP might be used to confirm the clinical diagnosis in a patient. There is no cure for HSPs; however, the resulting information from a genetic testing can be used for genetic counseling and prenatal/preimplantation genetic diagnosis.

Neuropathological studies suggest that HSPs are likely to occur due to degradation of the corticospinal motor neurons and axon degeneration (117, 118). The most important molecular mechanisms underlying the HSPs can be recruited from the 58 identified genes up to date: axon transport (SPG30 [KIF1A], SPG10 [KIF5A], SPG4 [Spastin], SPG20 [Spartin]), endoplasmic reticulum morphology (SPG3A [Atlastin], SPG4 [Spastin], SPG12 [reticulon 2], SPG31 [REEP1]), mitochondrial function (SPG13 [chaperonin 60], SPG7 [paraplegin], mitochondrial ATP6), myelination (SPG2 [Proteolipid protein], SPG42 [Connexin 47]), protein folding and degradation (SPG6 [NIPA1], SPG8 [K1AA0196], SGP17 [BSCL2], SPG18 [ERLIN2]), vesicle transport and membrane trafficking (SPG47 [AP4B1], SPG48 [KIAA0415], SPG50 [AP4M1], SPG51 [AP4E], SPG52 [AP4S1], VSPG53 [VPS37A]), and lipid metabolism (SPG28 [DDHD1], SPG35 [FA2H], SPG39 [NTE], SPG54 [DDHD2], SPG56 [CYP2U1]) (40, 116). Exploring new genes related to interacting molecular pathways and their evaluation in animal models will allow the exploration of disease mechanisms and potential treatments.

3.2.2 Troyer Syndrome

Troyer syndrome (OMIM #275900) is classified as a complicated HSP inherited in an autosomal recessive fashion. The most common characteristics of Troyer syndrome are spastic paraparesis, distal amyotrophy, progressive muscle weakness, subtle skeletal abnormalities, mild developmental delay, learning disorders, dysarthria and mood swings. Other characteristics might include hyperreflexia, pseudobulbar palsy (119).

3.2.2.1 Previously Identified Families with Troyer Syndrome

3.2.2.1.1 Old Order Amish

Troyer syndrome was first identified in 1967 in three individuals with Old Order Amish origin in Ohio, USA (119). 18 additional individuals with Troyer syndrome in the same Amish population have been reported up to date (120). The Old Order Amish population practice endogamy and they have large families, which is suitable to study recessively inherited conditions. These individuals were from different families; however, all of them were traced back to a common ancestor. The ages of patients were between 2 and 63 years, and the most common feature they share was delayed walking and talking. Distal amyothropy was present in all individuals older than 13 years (except a 7 year-old patient). Learning difficulties, difficulty at school and emotional lability were also reported in the patients. Most of the patients had spastic dysarthria. The patients exhibited mild skeletal abnormalities including short stature, small feet with pes cavus, and hammer toes. Brain magnetic resonance images (MRI) showed white matter abnormalities in affected individuals.

SPG20 c.1110delA frameshift mutation has been identified in two Amish families (121, 122). This frameshift mutation resulted in loss of protein expression in cells isolated from affected individuals (122).

3.2.2.1.2 Omani

Troyer syndrome was also described in two related Omani families. Their common symptoms were dysarthria and delays in motor and cognitive development, difficulties in walking, mild spastic gait, short stature and hand and foot abnormalities

including brachydactyly, hammer toes, and pes cavus (123). Similar to the Amish families, the patients of Omani family had poor performance in school.

SPG20 c.364_365delAT mutation was identified as the cause of Troyer syndrome in the two related Omani families (123). This frameshift mutation also results into lack of expression of spartin protein (123).

3.2.2.1.3 Other families with Troyer-like Features

A Kuwaiti and an Austrian family were reported with features similar to that of Troyer syndrome. However, sequencing *SPG20* in both of these families did not reveal any damaging mutations which suggested a different genetic etiology (120, 124, 125).

3.2.3 Aim of this study

In this study, we report a family in which two of the three siblings exhibit several neurological symptoms similar to that of Troyer syndrome. Whole exome sequencing was performed to two affected siblings in the family in order to identify the causative mutation.

3.3 Materials and Methods

3.3.1 Subjects

The family is of Turkish origin. The proband and his informative family members were evaluated at Hacettepe University Medical School. This study was approved by the ethics committees of all participating universities, and all individuals provided informed consent. 346 healthy controls from central Anatolian region of Turkey were obtained from Bilkent University for genetic analysis.

3.3.2 DNA isolation

DNA of the affected subjects and relatives were extracted from blood using Nucleospin Blood Kit (Macherey-Nagel) according to manufacturer's protocol. Two affected siblings (subjects IV:1 and IV:2) were selected for whole exome sequencing (Table 3.1).

Table 3.1 Concentration and quality of DNA extracted from patients for whole exome sequencing from ET-4 family

Sample ID	Concentration (ng/µl)	$A_{260/280}$	$A_{260/230}$
IV:1	177	1.8	1.6
IV:2	183.8	1.8	1.6

3.3.3 Library construction and whole exome sequencing

Library construction and sequencing was carried out according to the protocols described previously (126). Briefly, the genomic DNA isolated from two affected individuals was randomly sheared into 200-300 bp fragments which is followed by end repair, A-tailing and indexed paired-end adapter ligation. Exomes were captured by SeqCap EZ Exome v2 (Niblegen, Roche), hybridized to biotynylated probes, amplified, and purified. DNA libraries were quality checked at this step by Agilent 2220 TapeStation Instrument (Figure 3.1). Libraries were sequenced on an llumina HiSeq2500 platform.

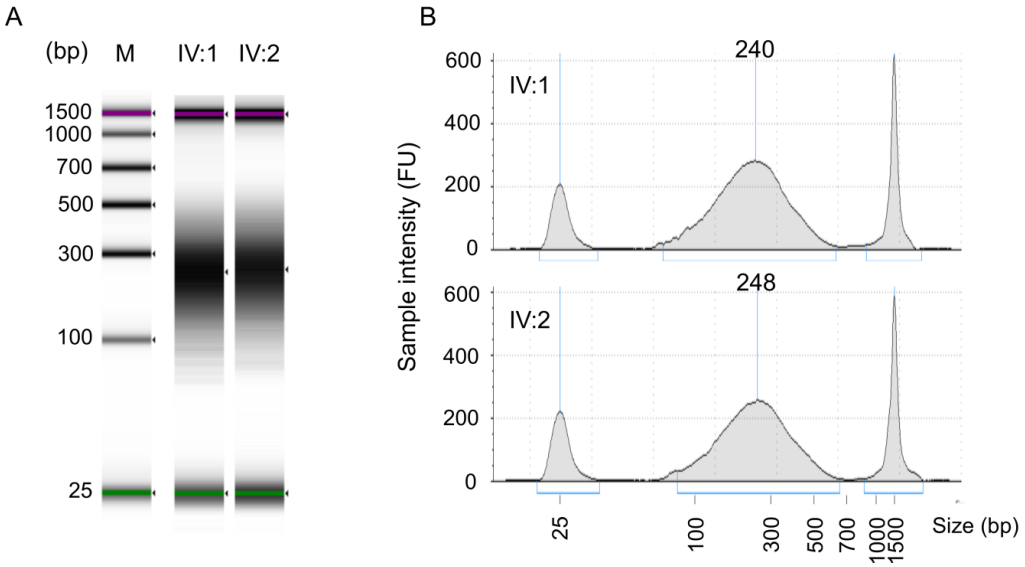

Figure 3.1 Quality control of post captured amplified whole exome libraries of ET-4 family. *(A)* Gel image of post-captured DNA library. Purple and red lines are intrinsic upper and lower markers, respectively. *(B)* Histogram representation of the results from panel *A*. bp: base pair, M: marker.

3.3.4 Bioinformatics

Reads were mapped to the human reference genome (hg19) using Burrows-Wheeler Aligner (BWA, v0.6.1-r104) (92). SAMtools v0.1.18 was used to remove PCR duplicates, sorting and indexing of the alignments (93). Indel realignments and base quality score recalibration (BQSR) were done with Genome Analysis Tool Kit (GATK, v3.0-0-g6bad1c6) (94). Genotypes were called and filtered using GATK Unified Genotyper and Variant Filtration tools. Variants were annotated using University of Washington King Lab's in-house pipeline. Artifacts and common variants were excluded using dbSNP v138, the National Heart, Lung, and Blood Institute (NHLBI) Exome Sequencing Project, the 1000 Genomes Project, and 700 exomes previously sequenced in our lab. Missense variants were evaluated for their functional impact using SIFT ($\leq$ 0.05) (46), PolyPhen2 ($\geq$ 0.8) (47) and MutationAssessor ($\geq$ 1.95) (48) prediction tools.

3.3.5 Sanger sequencing and population screening

Candidate variants were genotyped in 14 informative family members by capillary sequencing to test for co-segregation. Primers were designed using Primer3 (95) (Table C2). The PCR products were Sanger sequenced and Sanger traces were analyzed with CLCBio Main Workbench software package (CLCBio Inc.).

3.3.6 Population screening

TaqMan genotyping assay (*Life Technologies*) was used for population screening of SPG20 p.G580R in 346 healthy Turkish controls.

Table 3.2 Clinical characterization of ET-4 family and comparison with previously published studies

Features	**IV:1**	**IV:2**	**Omani**	**Amish I**	**Amish II**
SPG20 mutation	p.G580R	p.G580R	c.364_365_delAT	c.1110delA	c.1110delA
Age (y)	14	11	Mean 16.6	Mean 12.25	Mean 40.7
Sex	M	F			
Height (cm)	154	139	Short	Short	Short
%	10	10-25			
OFC (cm)	50.5	49	Normal	Normal	Normal
%	50-75%	25-50%			
Weight (kg)	39	31	N/A	N/A	N/A
Developmental milestones and cognition					
Talked at (y)	2.5	1.5	Late (100%)	Late (87.5%)	Late (80%)
Walked at (y)	1	1	Late (67%)	Late (87.5%)	Late (80%)
Poor school performance	+	+	+ (80%)	+ (100%)	+ (83.3%)
Emotional lability	-	-	-	+ (62.5%)	+ (92.3%)
Neurological exam					
Dysarthria	+	+	+ (100%)	+ (100%)	+ (100%)
Tongue dyspraxia	+	-	+ (80%)	Often	Often
Distal amyotrophy	-	-	+ (67%)	+ (62.5%)	+ (100%)
Hyperreflexia upper	+	+	+ (33%)	+ (12.5%)	+ (91.7%)
Hyperreflexia lower	+	+	+ (67%)	+ (100%)	+ (100%)
Ankle clonus	-	-	+ (50%)	+ (25%)	+ (44.4%)
Clumsy, spastic gait	+	+	+ (100%)	+ (100%)	+ (100%)
Dysmetria	+	+	+ (50%)	+ (62.5%)	+ (100%)
Extensor plantar reflex	+	-	N/A	+ (62.5%)	+ (81.8%)
Tremor	Action	Action	N/A	N/A	N/A
Tremor onset (y)	4	4	N/A	N/A	N/A
Skeletal Abnormalities					
Overbite	-	-	+ (100%)	No	No
Hypertelorism	-	-	+ (83%)	No	No
Hand/foot abnormalities	-	-	+ (100%)	+ (100%)	+ (100%)

OFC: occipitofrontal circumference, N/A: not available. *clinodactyly, camptodactyly, hypoplastic 5th middle phalanges

3.4 Results

3.4.1 Clinical features of the family

The ET-4 family originates from the south-eastern Anatolia of Turkey. The proband (subject IV:1) was referred to Hacettepe University Medical School because of his severe action tremor at age 14 years. He was born at term with a birth weight of 3.2 kg and head circumference of 35.5 cm. There was not any history of exposure to teratogens or infection during pregnancy. His initial developmental milestones were slightly delayed. He walked at age 12 months, and talked at age 30 moths. His tremor was first recognized at age of 4 years and slowly progressed over time. His tremor was very severe at the time of examination that he needed special help for his daily activities, such as eating and drinking. Physical examination upon the first visit revealed weight of 39 kg (90%), height of 154 cm (10%), and OFC of 50.5 cm (50-75%). Neurological examination showed moderate to severe tremor and clumsy, spastic gait with mild ataxia. He had dysarthria, tongue dyspraxia, hyperreflexia in upper and lower limbs, dysmetria, and positive Babinski sign (Table 3.2). He did not have any skeletal abnormalities (Figure 3.2). His IQ was 70. He could not attend to mainstream school, and was receiving special education. Brain MRI scans showed bilateral vermian cerebellar atrophy, bilateral cerebral symmetric hyperintensities and patchy signal hyperintensity in the pons in T2 weighted images (Figure 3.3A, B). His 11 year old sister (subject IV:2) was also similarly affected with age at onset of 4 for tremor. Her developmental milestones were normal compared to her unaffected sister. She had dysarthria, upper and lower hyperreflexia, dysmetria and action tremor (Table 3.2). Her MRI scans showed bilateral thalamic atrophy, bilateral posterior dominant periventricular and ventrolateral thalamic hyperintensities (Figure 3.3C, D). Both

patients were able to walk. They had a 4 year old healthy sibling. Their parents were first cousins. The mother's first pregnancy product died *in utero* in the 6th week of gestation with no apparent cause.

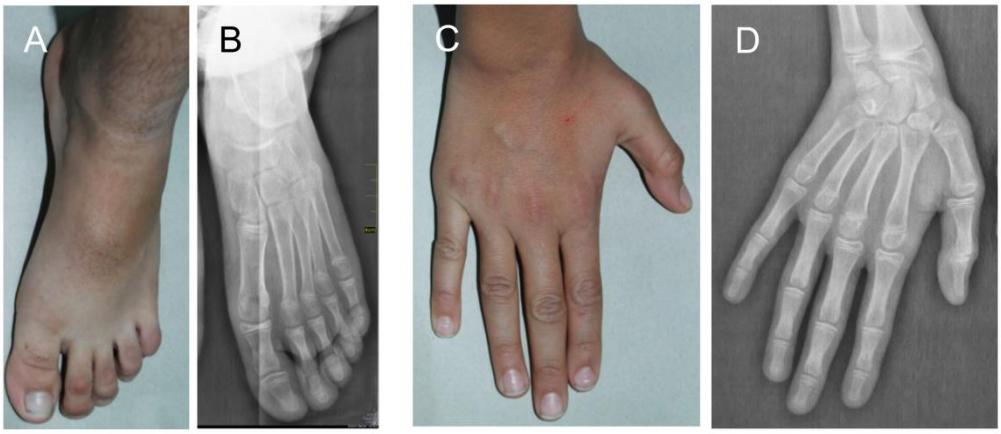

Figure 3.2 Morphological and radiological analysis of extremities of subject IV:1. Subject IV:1 with Troyer syndrome does not have any foot (A, B) or hand (C, D) skeletal abnormalities.

3.4.2 Gene discovery

The exome sequence data of two affected siblings were summarized in Table 3.1. Coverage of targeted genomic regions was 33.4X for IV:1 and 53.9X for IV:2 (Figure E2). Percent of targeted regions covered at least 4X was 94% for IV:1 and 97% for IV:2. A detailed statistics about the results of exome sequence experiment is given at Table 3.3. Whole exome sequencing revealed 12 homozygous rare variants, three of which are damaging (Table 3.4; Table 3.5). All of these three mutations were segregating with the disease in ET-4 family. Among these genes, *C17orf70* encodes a fanconi anemia associated protein (FAAP100) which is a component of fanconi anemia core complex (127). The fanconi anemia phenotype was ruled out by clinical examination of the patients. CSNK1A1L p.Q198K mutation was identified in a retro-

gene originated from *CSNK1A1* which is highly expressed in testis (RetrogeneDB). The third mutation was SPG20 (spartin) p.G580R at chr13:36,878,765 C>T (NM_015087). SPG20 p.G580R mutation was predicted to be damaging by prediction tools Polyphen2 (score 1.0), SIFT (P = 0.0) and MutationAssessor (score 2.150) (Table 3.5). Based on the phenotypic overlap between families with SPG20 mutations and this Turkish family, we focused on SPG20 p.G580R mutation as the cause of Troyer syndrome in the kindred.

SPG20 p.G580R mutation was inherited in an autosomal recessive fashion with the disease in the family (Figure 3.4A, B). The mutation lies in a highly conserved PDZ domain of spartin protein (Figure 3.5A, B). SPG20 p.G580 mutation was not detected in 346 healthy Turkish controls 650 in-house exomes, and was absent from the population database, including dbSNP38, 1000 Genomes Project, NHLBI Exome Sequencing Project and ExAC V0.2.

3.5 Discussion

In this study, we report the third mutation identified in *SPG20* in a Turkish family with Troyer syndrome. Loss of function mutations in *SPG20* have previously been identified in two different populations with Troyer syndrome; Amish and Omani (122, 123, 128). After the characterization of the first Troyer syndrome cases in the Amish population, several Troyer-like syndromes have been identified in other populations (124, 125); however, sequencing of *SPG20* in these families did not reveal any damaging mutations (120). We compared the clinical features of the individuals in the Turkish family with that of the previously reported Amish and Omani families with

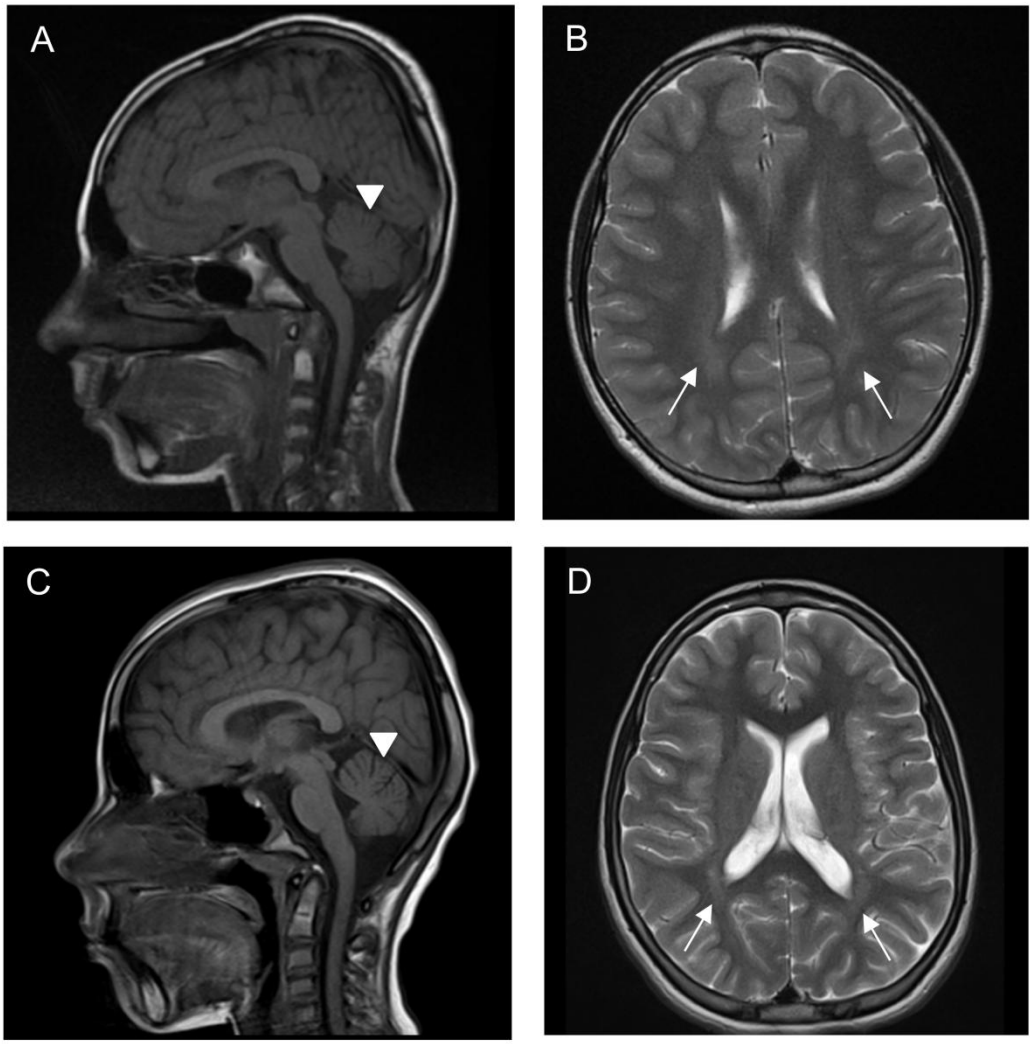

Figure 3.3 Brain MRI scans of affected subjects. Thalamic atrophy (white arrows) and mild vermian cerebellar atrophy (arrowheads) of subjects IV:1 *(A)* and IV:2 *(C)* in axial T1 weighted MRI sequences. Bilateral periventricular posterior dominant hyperintensities (white arrows) of subjects IV:1 *(B)* and IV:2 *(D)* in axial T2 weighted MRI sequences.

Table 3.3 Results of whole exome sequencing of DNA from two affected relatives from family ET-4

Fetures	IV:1	IV:2
Total reads	63,885,397	13,0874,348
Average coverage	33	54
% coverage at 4X	94	97
% coverage at 20X	67	83
Coding variants	19,410	19,617
Shared by all 2 exomes	16,069	
Rare variants[a]	240	
Homozygous in 2 exomes	12	
Predicted damaging to protein function[b]	3	

Abbreviations.

[a]Filtered by dbSNP138 common SNPs and MAF ≥ 0.001 in EVS or 1000G variants.

[b]Truncating mutations, splice site mutations predicted to lead to altered transcripts, and missense variants with all of Polyphen2 score ≥ 0.8, SIFT score ≤ 0.05 and MutationAssessor score ≥ 1.95 were defined as damaging. Both heterozygous and homozygous variants were included.

Table 3.4 Homozygous rare variants shared by IV:1 and IV:2

Chr	Coordinate	Ref	Var	Type	Gene	Protein	cDNA	PPH2	SIFT	MA	Reads (Total|Variant) IV:1	Reads (Total|Variant) IV:2
2	183,791,545	A	G	Missense	NCKAP1	M1090T	c.T3269C (exon30)	0.307	0.172	1.040	33 | 33	53 | 53
2	242,684,234	C	T	Silent	D2HGDH	I131I	c.C393T (exon5)	-	-	-	29 | 29	51 | 51
4	54,327,703	G	A	Silent	LNX1	G554G	c.C1662T (exon9)	-	-	-	43 | 42	96 | 96
4	54,244,053	G	C	Silent	FIP1L1	G16G	c.G48C (exon1)	-	-	-	25 | 25	64 | 64
6	135,265,012	C	T	Silent	ALDH8A1	A77A	c.G231A (exon2)	-	-	-	23 | 23	26 | 25
9	71,080,024	G	A	Missense	PGM5	M353I	c.G1059A (exon7)	0.257	0.015	1.245	48 | 48	76 | 76
13	**37,678,802**	**G**	**T**	**Missense**	**CSNK1A1L**	**Q198K**	**c.C592A (exon1)**	**1.000**	**0.000**	**4.595**	**82 | 82**	**134 | 134**
13	**36,878,765**	**C**	**T**	**Missense**	**SPG20**	**G580R**	**c.G1738A (exon9)**	**1.000**	**0.000**	**2.150**	**31 | 31**	**30 | 30**
17	**79,511,038**	**G**	**A**	**Missense**	**C17orf70**	**R807C**	**c.C2419T (exon7)**	**1.000**	**0.000**	**2.210**	**13 | 13**	**24 | 24**
17	75,478,306	C	T	Missense	SEPT9	R156W	c.C466T (exon2)	0.997	0.002	1.795	9 | 9	19 | 19
17	79,639,666	C	T	Missense	CCDC137	R268W	c.C802T (exon6)	0.996	0.016	1.320	17 | 17	23 | 23
17	72,916,421	G	C	Silent	USH1G	A67A	c.C201G (exon2)	-	-	-	15 | 15	26 | 26

Bold represents variants predicted to be damaging.

Table 3.5 Rare damaging variations identified in two affected siblings IV:1 and IV:2

Chr	Start	Ref	Var	Gene	Effect	PPH2	SIFT	MA	EVS	1000G
13	36,878,765	C	T	SPG20	G580R	1.000	0.000	2.150	-	-
13	37,678,802	G	T	CSNK1A1L	Q198K	1.000	0.000	4.600	-	-
17	79,511,038	G	A	C17orf70	R807C	1.000	0.000	2.210	0.00007	-

Abbreviations: Chr: chromosome, Ref: reference base, Var: variant base, PPH2: Polyphen2, MA: MutationAssessor, EVS: Exome variant server, 1000G: 1000 genomes project.

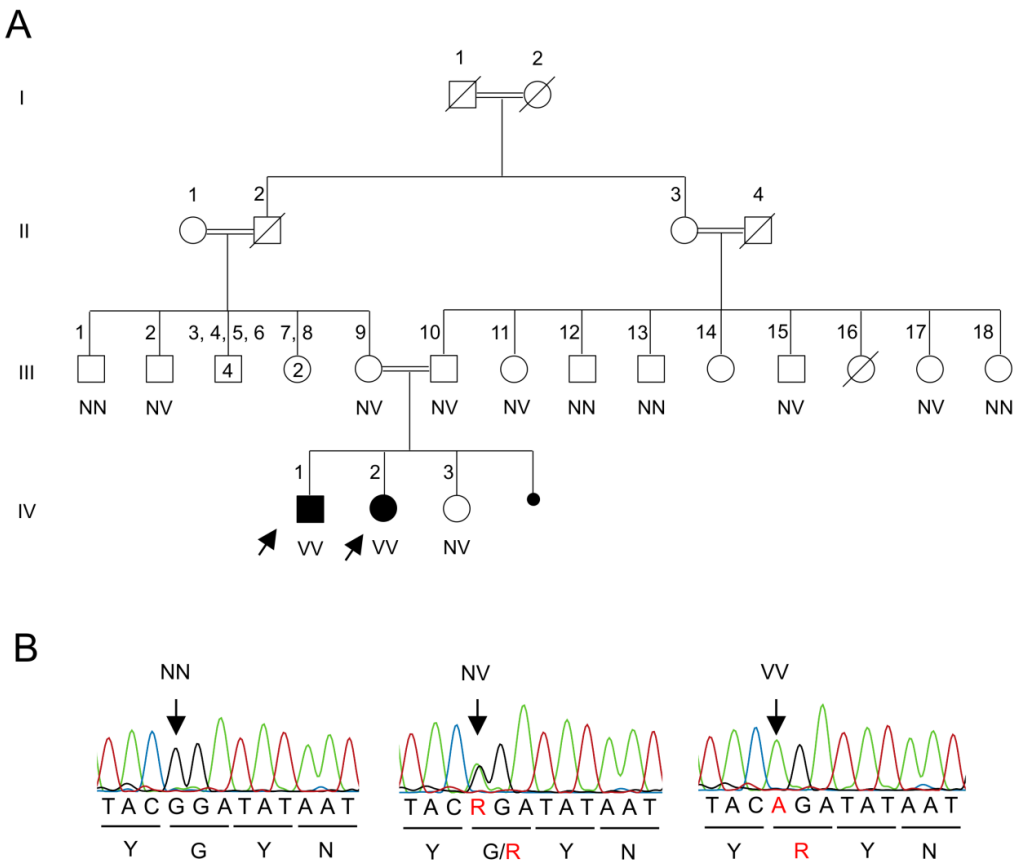

Figure 3.4 Pedigree of family ET-4 and segregation of the *SPG20* mutation. *(A)* Pedigree of ET-4 family segregating Troyer syndrome, with genotypes at p.G580. Filled and unfilled symbols represent affected and unaffected individuals, respectively. N indicates the wildtype allele, glycine; and V indicates the mutant allele, arginine at SPG20 p.G580R. Individuals who underwent exome sequencing (IV:1 and IV:2) are represented with arrows. *(B)* Sanger traces of an unaffected wildtype individual, an unaffected heterozygous parent, and an affected homozygous patient. The arrows show the mutation site.

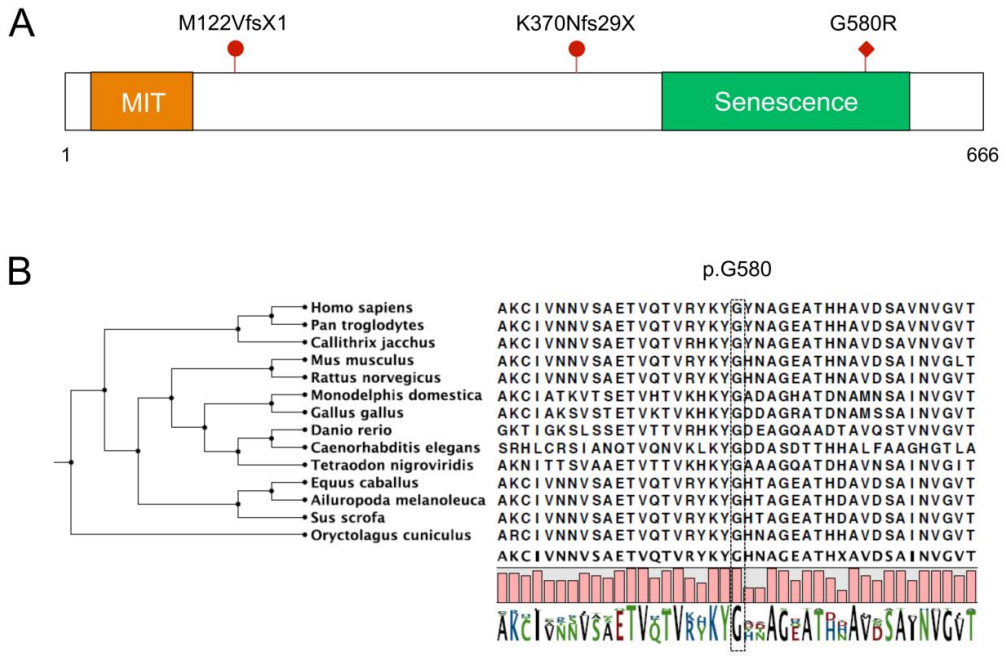

Figure 3.5 Spartin protein and its sequence homology among species. *(A)* Full length spartin consists of a microtubule-interacting and trafficking molecule (MIT) domain (aa 16-94), and a plant related senescence domain (aa 426-613). The positions of the mutations identified in spartin are shown. *(B)* Sequence homology of spartin protein.

with *SPG20* mutations (Table 1). Up to date, 21 individuals with Troyer syndrome were reported from the same Old Order Amish population (120). The ages of patients ranged from 2 to 63 years. Since the symptoms of the Troyer syndrome worsens with age, we sub-grouped the affected individuals in the Amish population according to their ages: Amish I group (< 24 y, mean age 12.25 y), and Amish II group (≥ 24 y, mean age 40.7 y). The ages of Omani patients ranged from 4 to 27 years with mean age of 16.6 years. Table 1 clearly shows that individuals in Amish II group have the most severe phenotypes. The clinical features of the Turkish family resembles that of the age-matched Amish I populations. The severity of the clinical features of Omani patients lie in between Amish II and Amish I populations, and is slightly severe than that of Turkish population. As opposed to Omani and Amish families, Turkish individuals do not have any skeletal abnormalities or distal amyotrophy. According to the reports, distal amyotrophy was not present in young subjects, and it was more severe in older cases (120, 123); which explains the lack of this symptom in the young Turkish individuals.

SPG20 encodes a 666 amino acid spartin protein which is widely expressed in humans. It is a multifunctional protein consisting of a microtubule interacting and trafficking (MIT) domain and a plant related senescence domain (Figure 3.5). Spartin was reported to localize to several different cellular compartments such as neurites, endosomes, midbodies, lipid droplets (LDs), mitochondria, and trans-Golgi network in differentiated neurons (129-132). Spartin interacts with endosomal ESCRT-III protein IST1 with its MIT domain (131). The ESCRT-III complex is a major part of ESCRT machinery which enables membrane remodeling including multivesicular body (MVB) biogenesis and cellular abscission (131). In a cell, ubiquitin tagged misfolded or damaged proteins are located to MVBs, which fuse with lysosomes to destroy these proteins (133). When ESCRT machinery does not work properly, these damaged

proteins aggregate and lead to neurodegenerative disorders. SPG4 protein spastin also interacts with the ESCRT -III protein IST1 which suggests a shared role of ESCRT machinery in different forms of HSP. Spartin has a proline rich PPxY sequence motif which interacts with E3 ubiquitin ligases AIP4 and AIP5 (131, 134). Spartin also takes role in many different cellular processes including the endocytosis and transport of the epidermal growth factor receptor (EGFR), and cytokinesis (135).

Several biochemical studies and experiments in model organisms have been performed to dissect the effect of mutations in *SPG20* in HSP phenotype. Quantitative PCR and *in situ* hybridization analysis showed that *Spg20* has its most specific expression in the limbs, face and forebrain during early morphogenesis which correlates with the phenotypic abnormalities observed in individuals with Troyer syndrome who lack spartin protein (123). Spartin protein is expressed in Turkish Troyer syndrome patients with SPG20 p.G580R mutations, which might explain the normal skeletal features observed in these patients. *Spg20*$^{-/-}$ mice showed significant gait phenotype together with defects in axon branching in cerebral cortical neurons, LD maintenance, cytokinesis, and BMP signaling (136). These defects are associated with several Troyer syndrome abnormalities. The Troyer syndrome model in *Drosophila* showed that flies expressing mutant spartin have defects in the ability of climbing which was rescued by the neuronal expression of spartin (137). Moreover, aged adult spartin mutant flies displayed neurodegeneration which was found to be a cause of increased BMP signaling. BMP signaling is essential for neuronal development and distal axonal function (138). Defects in this pathway have previously been identified in some of the other HSP proteins, such as atlastin-1 (*SPG3A*), spastin (*SPG4*), and NIPA (*SPG6*) (138).

Spartin ortholog in *C. elegans* (F57B10.9) is expressed in the nervous system (wormbase). As a future study, phenotypic effect of p.G580R mutation in *C. elegans* will be evaluated. *C. elegans* have successfully been used as a model organism due to its simple genetics. Their transparent body makes the worm anatomy easily observable through light microscopy. Moreover, they are really easy to cultivate and genetically manipulate. Therefore, *C. elegans* is an ideal system to study the molecular bases of human diseases like HSPs.

Our results show that Troyer syndrome might be caused by a missense mutation in spartin protein. The clinical findings in these three studies, including this study, suggest that Troyer syndrome starts with developmental abnormalities, has slow but definite progression which supports neurodegeneration. Further functional studies will be conducted in order to reveal the effects of p.G580R missense mutation in SPG20 related biological pathways and BMP signaling.

Chapter 4

4 Conclusion and Future Perspectives

4.1 Research Summary

In summary, this thesis focused on the genetic analysis of two clinically complex neurodegenerative disorders: Essential tremor, one of the most prevalent movement disorders; and Troyer syndrome, a very rare disorder with a highly severe phenotype. Whole exome sequencing with the following pedigree analysis identified causative mutations for both disorders. A genotype-phenotype correlation was shown in both diseases. The second chapter shows that individuals with heterozygous HTRA2 p.G399S develop essential tremor with a mean age at onset of 40 y. On the other hand, in individuals with two mutant alleles, essential tremor tended to appear earlier in life, progressing to Parkinson's disease when individuals reach middle age. The third chapter describes a family with Troyer syndrome with homozygous SPG20 p.G580R mutations. Loss of function mutations in SPG20 have already been known to cause Troyer syndrome with spastic gait, dysarthria, distal amyotrophy, mild developmental delay, learning disorders, and subtle skeletal abnormalities. Nevertheless, individuals carrying homozygous SPG20 missense mutations do not develop skeletal abnormalities.

4.2 Unraveling the genetic bases of complex neurodegenerative disorders

Traditional strategies had identified many disease loci and genes; however, the genetic basis of complex neurodegenerative disorders remained to be elusive. Whole exome sequencing allows us to identify the causative mutations in previously undiagnosed or clinically complex conditions. The advantages of consanguineous families have been used for years to identify disease causing mutations through homozygosity mapping strategies. This leaded to the identification of hundreds of causative mutations for autosomal recessive disorders. The studies in this thesis showed that consanguineous families also help us to identify disease causing mutations for complex neurodegenerative diseases; i.e., consanguineous marriages introduce individuals with common disorders with a much severe phenotype due to homozygosity, allowing the identification of causative dominant mutations with dosage effect.

4.3 Application to clinical practice

Gene identification studies gain their most important roles in clinical medicine. The major role of disease gene identification studies is to obtain an understanding of the genetic bases of diseases. The application of whole exome sequencing to clinical diagnosis is already being used with high success rates (139, 140). Clinical diagnosis through sequencing technologies allows the recognition of the risk of recurrence in subsequent pregnancies, and provides guidance and prognosis. For example, the NIH Undiagnosed Diseases Program aimed to provide answers to patients with an undiagnosed condition using high density SNP arrays, or whole exome and whole

genome sequencing (139). In the first year, 326 patients were accepted where 53% of them had an unknown neurological disorder. 24% of all participants were diagnosed on clinical, biochemical, pathologic or molecular grounds (139). This saves patients from the pain, cost and time of additional test, and provide them a definite diagnosis (141).

In addition to clinical diagnosis, the information obtained from the genomic data will be used as a source of personalized medicine which aims "to provide the right treatment to the right person at the right time" (142). As the technology improves, sequencing becomes affordable to gain an understanding of the pathophysiological mechanisms and biochemical pathways underlying disorders (35). Understanding of the biology of disease process can be obtained by identifying multiple associated genes that affect one biological pathway. Detection of mutations in proteins that are known to be involved in protein degradation, mitochondrial and endoplasmic reticulum homeostasis, and axonal transport strongly supports that defects in these mechanisms can result in neurodegeneration. Moreover, chronic exposure to certain toxic chemicals can trigger the defects in these pathways and can also result in neurodegeneration. The main aim would be to develop suitable therapeutic targets for these common pathways. All of these mechanisms leading to neurodegeneration can act synergistically for a single neurodegenerative disorder. Most proteins serve many diverse functions adding them into more than one pathway. When considering the treatments for neurodegenerative disorders, it is important to know which one of these pathways to target at which particular stage of the disease (141). As a future perspective, scientists should focus on the identification of new genes in shared pathways; and their analysis in animal models will deepen our knowledge in the pathophysiological consequences of neurological dysfunctions.

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Appendices

Appendix A

Accession codes

Accession codes for HTRA2 orthologs

Ailuropoda melanoleuca:	XP_002921511
Callithrix jacchus:	XP_002757664
Equus caballus:	XP_001500096
Gallus gallus:	XP_423666
Homo sapiens:	NP_037379
Monodelphis domestica:	XP_001376681
Mus musculus:	AAH49880
Oryctolagus cuniculus:	XP_002709758
Pan troglodytes:	NP_001267074
Rattus norvegicus:	NP_001100069
Sus scrofa:	XP_005674479

Accession codes for SPG20 orthologs

Ailuropoda melanoleuca:	XP_002928833
Callithrix jacchus:	XP_008996381
Caenorhabditis elegans:	F57B10.9
Danio rerio:	NP_001107130.1
Equus caballus:	XP_005601198
Gallus gallus:	XP_417098.1
Homo sapiens:	NP_055902
Monodelphis domestica:	XP_007495391
Mus musculus:	NP_659144
Oryctolagus cuniculus:	XP_002720632
Pan troglodytes:	XP_003314171
Rattus norvegicus:	XP_006232427
Sus scrofa:	XP_005668402
Tetraodon nigroviridis:	CAG09018
Xenopus (Silurana) tropicalis:	NP_001123727.1

Appendix B

Regions identical by descent in 5 affected relatives of ET-1 family

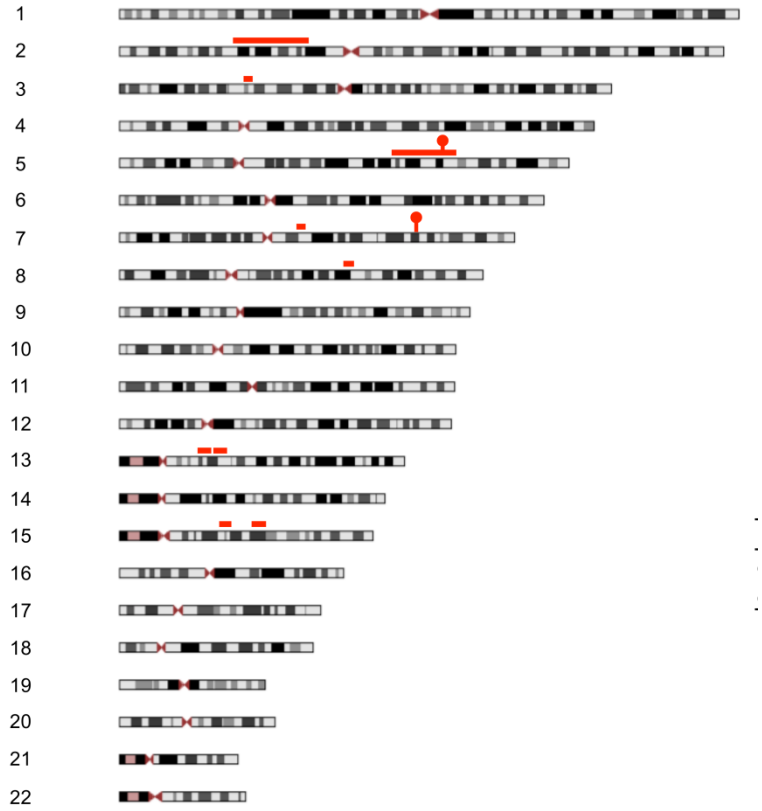

Figure B. Heterozygous or homozygous identical by descent regions with at least 3 MB in length, and shared by 5 affected relatives of ET-1 family. Red lines: IBD regions; red circles: homozygous regions

Table B. Heterozygous or homozygous identical by descent regions with at least 3 MB in length, and shared by 5 affected relatives of ET-1 family. HTRA2 p. G399S is on chromosome 2p (bold), in the longest region IBD shared by the 5 affected individuals based on our SNP analysis.

Chr	Start	End	Size (Mb)
2	**45,999,820**	**75,128,176**	**29.1**
3	50,113,035	53,332,706	3.2
5	109,518,552	135,395,203	25.9
7	71,597,460	74,715,724	3.7
8	90,352,835	94,092,917	3.7
13	31,642,859	37,144,848	5.5
13	38,350,194	43,421,683	5.1
15	40,549,618	45,142,419	4.6
15	53,016,092	58,635,583	5.6

Appendix C

Primer List

Table C1. Primers used for PCR amplification and haplotype analysis of ET-1 family.

Primer Name	Direction	Sequence
Segregation		
EML6	F	CTGAGCTTGGGTTTGGAGAA
	R	CAGATCCTGCACAGACTTGG
HTRA2	F	ATGCCTGGGTTTGGCTAATA
	R	CAACTGGGATTGGGTTCG
NUP210	F	GTGTGAAGAGACGGCAGTGA
	R	TGTGAGAGTGTCCTGGGTGA
UVSSA	F	AATGAAGATGGGAAGGCAGT
	R	AGTGGTAGCCCAAGGCAAG
TBC1D7	F	AAACTAACCCTCAGGCCAAC
	R	GGGTTTGGGATAAAGTTGTGAG
ALDOB	F	GCTTCCTTCTTTACTTGCCTTC
	R	GGGTCCCTCGCACTAATACA
SETX	F	GGCTCAGGACACTGACGAA
	R	CCAATGGCTGAAGATCCTGT
ANKRD33	F	CCCACATCAGTCTTGCTCCT
	R	GTGGCAGTCACAAGGTGGTC
LTK	F	TACAGGAGGGAGGAGGTGAA
	R	TCATCCACAGGTTAGGAGCA
GCOM1	F	GGCCATTTCCTTATGTTCCA
	R	GCTGTTTCTGAAGTGCCAAG
ANPEP	F	GGCTGGAGACTTTGTCCTTG
	R	AGCTCCTCCTCAAGGCTGTT
MYO1D	F	TGGAGCAATCTCAAAGAGGA
	R	CTAAGCAAGCAACCACCACA
PLA2G4C	F	CAGAAGTTCGTTGGATGTGG
	R	CCTGGTGGATGCTGGTTTAG
Coding regions		
HTRA2_1	F	GTCCTACTGTCCGCCTGCT
	R	CTGTGACCACGGCCTCATAC
HTRA2_2	F	TGTGGTGGAGAAGACAGCAC
	R	AGAGCTAACAATGCCGGATG
HTRA2_3	F	CGCTGAGGATTCAGACTAAGG
	R	CTTGGAAGGAAGGATGTCTCA
HTRA2_4	F	TCAGTGTGGGAAGGGTAGGT
	R	GACAGAAATGAGAACAAAGCTCA
HTRA2_5	F	TTGATGAGAGACTTGAGGTGGA

	R	AGGAGTCAGTGCTGGTGGTT
Regulatory regions		
HTRA2_3UTR	F	TGAGGCTCCTGCTCTGATTT
	R	AGCATGGGAATCTTTGCTC
HTRA2_5UTR1	F	CCTCGTGGAAGCACAGAATC
	R	GGACACAGGAGGTGGTGACT
HTRA2_5UTR2	F	TCACGGTGCTACAGGTGGTA
	R	GAGCGGCTCTTTGACTCG
HTRA2_5UTR3	F	CGAGCAGTAGGAAGCAGTCA
	R	AGCAGGCGGACAGTAGGAC
STRs		
HTRA2_74.259	F	FAM-GGGAGGAGTTGTGAGAATGC
	R	GTTTCTTAGCACACAAGAACCAGGTCA
HTRA2_74.324	F	HEX-AGCAGGTCACAGAATAGCATGT
	R	GTTTCTTTTTAGATGACTACCCAGACATTTG
D2S2977	F	FAM-GGCAGCACAGTGACAGATAA
	R	GTTTCTTCTTCCCCAATCAACTCTCCT
D2S1394	F	FAM-GGCATCTTTATCCTTAGCCC
	R	GTTTCTTCGGGGTCTGCATTACAGTAT
SNPs		
rs11674899	F	TTCAAGTGACATTCAAGAGAGAGC
	R	GTGAGGGTGGAGAGACCAGA
rs3771770	F	AGTGGCCTGACTCTTGTCGT
	R	GACTACCTCCTCAGTGCTTTCA

Table C2. Primers used for PCR amplification ET-4 family.

Primer Name	**Direction**	**Sequence**
SPG20	F	TGTCCTGTTTGTGTTGCAGTT
	R	GCCAAAGTCTGGTTAAGGTAGAA
CSNK1A1L	F	CAGCCCACGACAGTAGTTCA
	R	ATGGGTACTGGGCGTCACT
C17orf70	F	CCTTAGTGAGCGACCCACAG
	R	CAGAGCAGGTTCTTGCCATT

Appendix D

Allelic discrimination plot

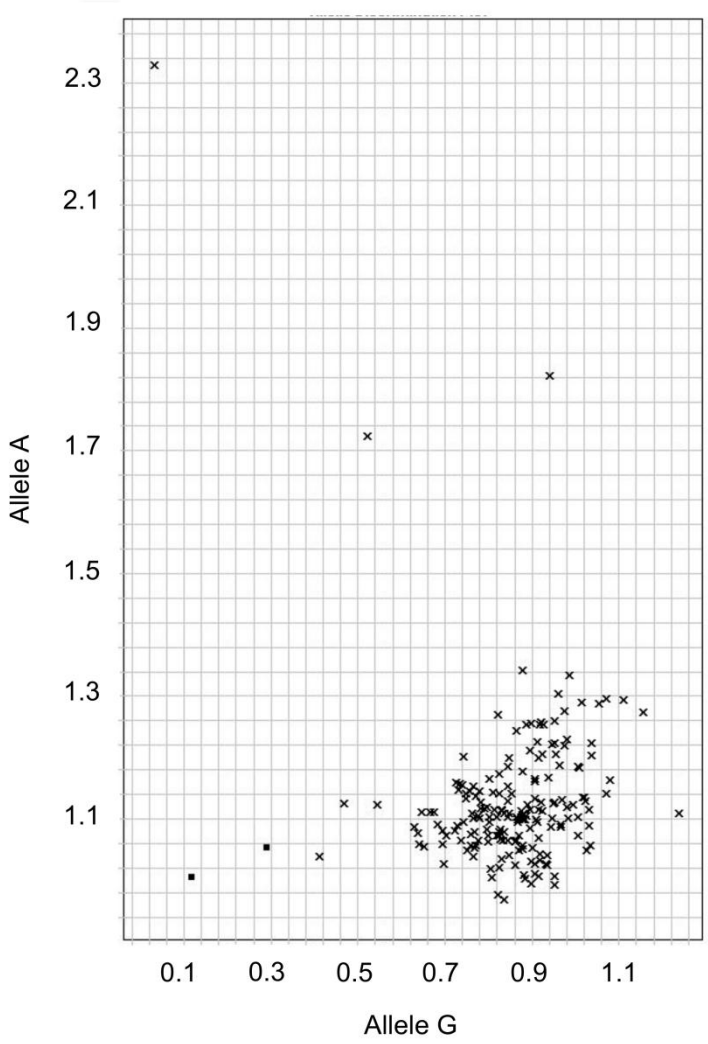

Figure D. Allelic discrimination plot of TaqMan genotype data of HTRA2 p.G399S variant in 183 individuals. The squares show NTC samples. Allele A homozygote clusters at the upper left corner, allele G homozygotes cluster at the lower right corner. Two heterozygotes cluster in the middle.

Appendix E

Percentage of targeted bases covered at particular depths.

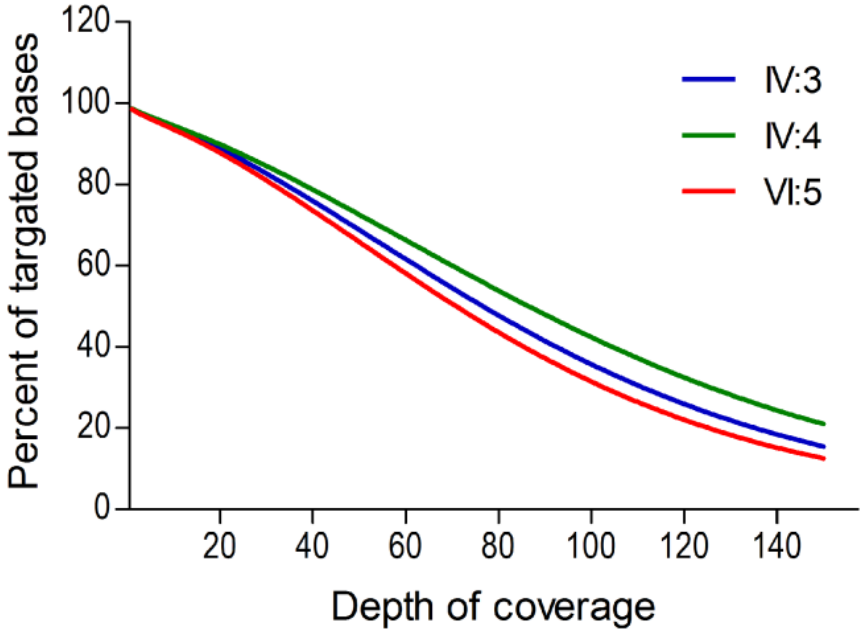

Figure E1. Percentage of targeted bases covered with whole exome sequencing of ET-1 family at particular depths.

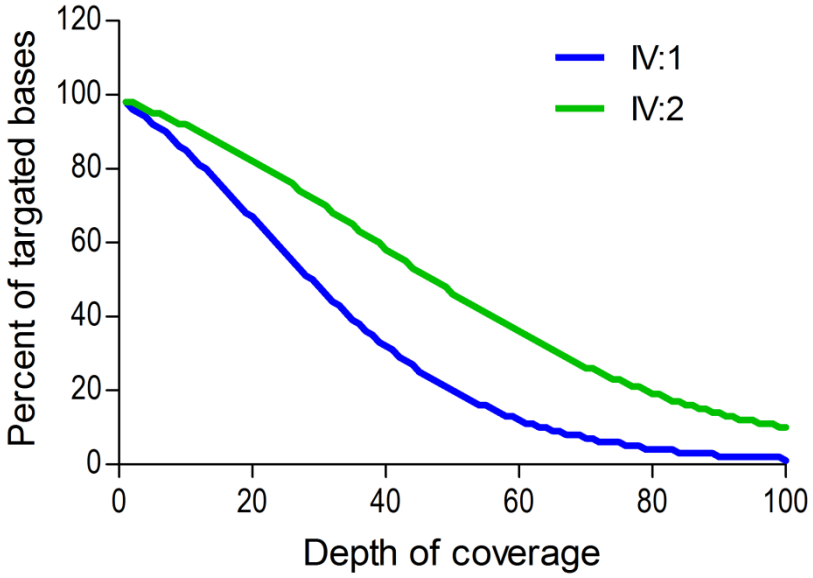

Figure E2. Percentage of targeted bases covered with whole exome sequencing of ET-4 family at particular depths.

Appendix F

Linkage to Previously reported *ETM* loci

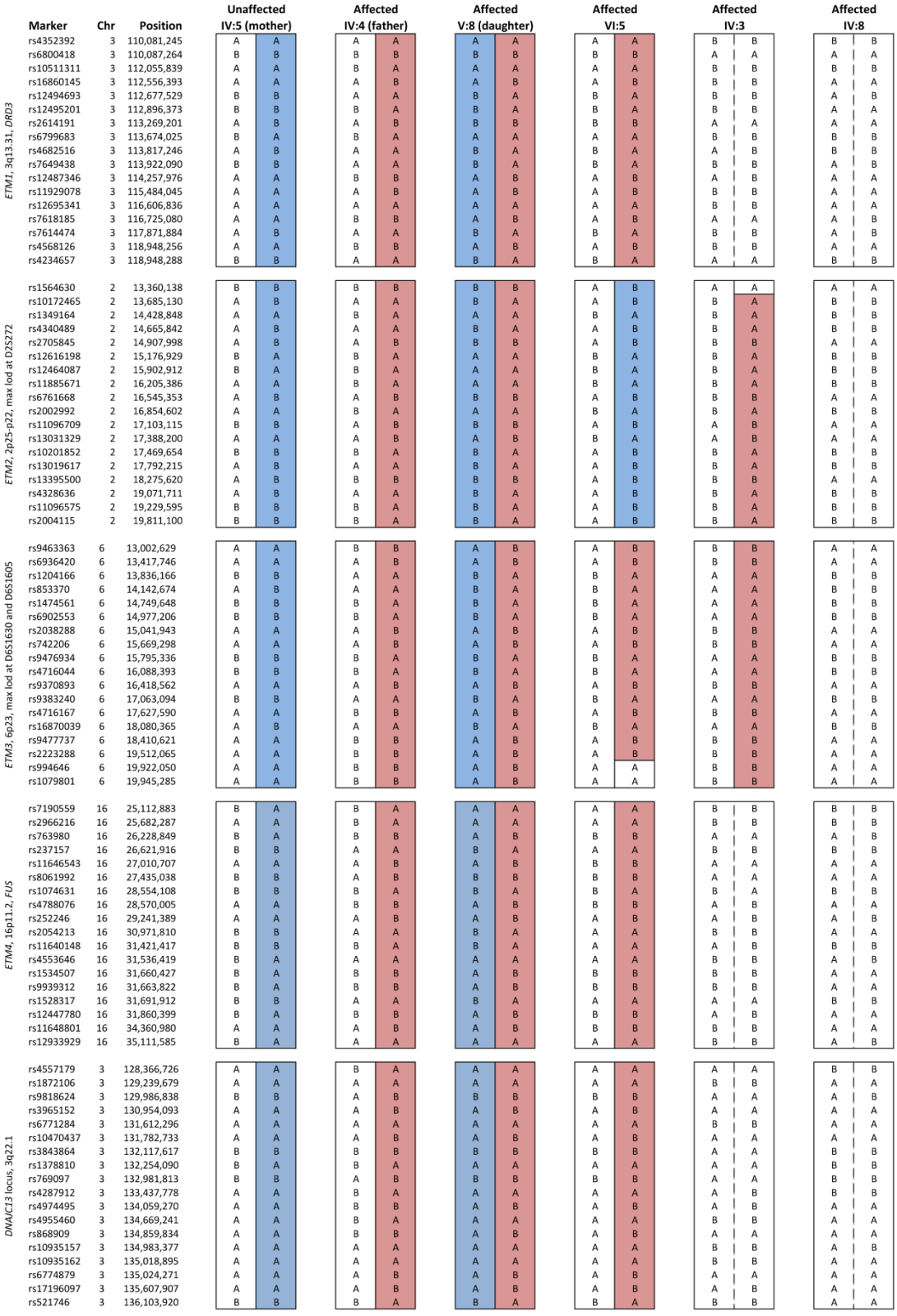

Figure F. Essential tremor in family ET-1 is not linked to *DNAJC13* or to genomic regions previously reported to be associated with essential tremor. At each region, the haplotype shared by the affected father-daughter pair IV:4 and V:8 is indicated in pink. At none of these regions was this haplotype also shared by the other affected family members.

Appendix G

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Publications

*: Equal contribution

1. **Unal Gulsuner H**, Gulsuner S, Mercan F.N, Onat O.E, Walsh T, Shahin H, Lee M. K, Dogu O, Kansu T, Topaloglu H, Elibol B, Akbostanci C, King M.-C, Ozcelik T, Tekinay A.B. "Mitochondrial serine protease HTRA2 G399S in a kindred with essential tremor and Parkinson disease", **Proc Natl Acad Sci USA**, 2014, 111(51):18285–18290

2. **Unal Gulsuner H**, Atalay Gengec N, Kilinc M, Erbil HY, Tekinay AB. "Osteoselection supported by phase separated polymer blend films" **J Biomed Mater Res A** 2015; 103(1):154-161

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