MOLECULAR ANALYSIS OF ALZHEIMER'S DISEASE AND FRONTOTEMPORAL DEMENTIA: A NOVEL MUTATION IN THE PRESENILIN1 GENE OF A TURKISH PATIENT WITH EARLY ONSET FAMILIAL ALZHEIMER'S DISEASE

by

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to my brothers

A. Levent Hocaoğlu & Kazım Koyuncu

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ABSTRACT

MOLECULAR ANALYSIS OF ALZHEIMER'S DISEASE AND FRONTOTEMPORAL DEMENTIA: A NOVEL MUTATION IN THE PRESENILIN1 GENE OF A TURKISH PATIENT WITH EARLY ONSET FAMILIAL ALZHEIMER'S DISEASE

Alzheimer's Disease (AD) and Frontotemporal Dementia (FTD) are the most common causes of dementia and death in developed countries. AD is clinically characterized by a progressive decline in cognitive functions. In most AD patients, the first symptoms start after the age of 60 years (late-onset or senile AD); however, rarely, the disease onsets earlier (early-onset or presenile AD). The other degenerative brain disease FTD has overlapping features with AD and a variable onset. In the framework of this thesis, six patients with clinically diagnosed early-onset AD, were screened for mutations in *Presenilin 1* (*PS1*) and *Presenilin 2* (*PS2*) genes, which cause AD when mutated. In addition, four FTD patients were analyzed for mutations in the exons 9-13 of the microtubule-associated protein *Tau* (*MAPT*) gene. Causative mutations in several patients remained unidentified, although the entire coding regions of PS1 and PS2 and the hotspot regions of *MAPT* were thoroughly investigated. This may have several reasons, including lack of family history and complicated differential diagnosis of early-onset familial AD (EOFAD) and FTD. As opposed to the above cases, a novel mutation was found in exon11 of a patient with autosomal dominant early onset AD. This is a <u>G</u>CC-<u>A</u>CC transition corresponding to an Ala396Thr substitution in the HL-VII domain of the PS1 protein. Its presence in the coding region of PS1 is thought to cause a change in the structure of the PS1 protein resulting in an alteration in amyloid precursor protein cleavage by constraining the structure of the protein. A better understanding of the complex etiology that underlies EOAD and FTD may be achieved through a multidisciplinary approach that combines clinical and neurophysiological characterization of these diseases with molecular investigations of genetic components.

ÖZET

ALZHEIMER HASTALIĞI VE FRONTOTEMPORAL DEMANSIN MOLEKÜLER ANALİZİ: BİR TÜRK ERKEN BAŞLANGIÇLI AİLESEL ALZHEIMER HASTASINDA PRESENİLİN1 GENİ ÜZERİNDE YENİ BİR MUTASYON

Alzheimer Hastalığı (AH) ve Frontotemporal Demans (FTD), gelişmiş ülkelerde en sık görülen demans ve ölüm nedenlerindendir. AH, klinik olarak bilişsel işlevlerin bozulmasıyla karakterize bir hastalıktır. AH olgularının çoğunda, ilk semptomlar 60 yaşından sonra (geç-başlangıçlı veya senil AH) ortaya çıkarken, nadir olarak hastalık daha erken başlayabilir (erken başlangıçlı veya presenil AH). Yine bir dejeneratif beyin hastalığı olan FTD, AH'ya benzer belirtiler göstermekle birlikte, başlangıç yaşı değişkendir. Bu çalışma çerçevesinde, erken-başlangıçlı AH klinik tanısı almış altı hastada, mutasyona uğradıklarında AH'ya neden olan *Presenilin 1* (*PS1*) ve *Presenilin 2* (*PS2*) genleri tarandı. Dört FTD hastasında ise, mikrotübülle ilişkili Tau proteini geninin (*MAPT*) 9-13. exonları, incelendi. PS1 ve PS2 geninin tüm kodlayıcı bölgeleri ve *MAPT* geninin en fazla mutasyon görülen beş ekzonu kapsamlı bir şekilde incelendiyse de, hastaların çoğunluğunda bu hastalıklara neden olan mutasyon tanımlanamadı. Bu durum, yetersiz aile öyküsü ve erken-başlangıçlı ailesel AH (EBAH) ile FTD'nin ayırıcı tanısındaki güçlüğe bağlı olabilir. Yukarıda sözü edilen olguların aksine, bir otozomal dominant EBAH olgusunda 11. ekzonda yeni bir mutasyon tanımlandı. Bu mutasyon, PS1 proteininin HL-VII bölgesi üzerinde Ala396Thr değişimine yol açan bir GCC-ACC değişimidir. *PS1*'in kodlayıcı bölgesinde bulunan bu mutasyonun, PS1 proteininin yapısını değiştirerek amiloid prikörsır protein kırpılmasında bozukluğa neden olduğu düşünülmektedir. EBAH ve FTD'ye neden olan karmaşık etkenlerin daha iyi anlaşılabilmesi, ancak bu hastalıkların klinik ve nörofizyolojik özelliklerini, genetik araştırma ile birleştirecek mültidisipliner bir yaklaşımla mümkün olabilir.

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LIST OF ABBREVIATIONS

3R Tau	Three repeat Tau
4R Tau	Four repeat Tau
A	Adenine
Aβ	β–amyloid protein (peptide)
AD	Alzheimer's Disease
Ala	Alanine
APP	β- Amyloid Precursor Protein
APS	Ammonium peroxosulphate
Arg	Arginine
bp	Base pair
BPB	Bromophenol Blue
C	Cytosine
cDNA	Complementary deoxyribonucleic acid
CT	Computerized Tomography
CTF	Carboxy terminal fragment
Cys	Cysteine
ddNTP	2', 3'-Dideoxynucleoside 5'-triphosphate
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	2'-Deoxynucleoside 5'-triphosphate
DSM-IV	Diagnostic and Statistical Manual-IV
EDTA	Ethylenedinitrilo-tetraacetate
EOAD	Early onset Alzheimer's Disease
EOFAD	Early onset familial Alzheimer's Disease
EtBr	Ethidium Bromide
EtOH	Ethanol
FTD	Frontotemporal Dementia
FTDP-17	Frontotemporal Dementia with Parkinsonism linked to Chromosome 17
G	Guanine
HL	Hydrophilic loop

kb	Kilobase
LOAD	Late onset Alzmeimer's Disease
M	Molar
MAPT	Microtubule associated protein Tau
Mg^{2+}	Magnesium
$MgCl_2$	Magnesium Chloride
MRI	Magnetic Resonance Imaging
mRNA	Messenger RNA
MT	Microtubule
NFT	Neurofibrillary tangles
NINCDS-ADRDA	National Institute of Neurologic and Communicative Disorders and Stroke and the AD and Related Disorders Association Work Group
NTF	Amino terminal fragment
OD	Optical Density
ORF	Open Reading Frame
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PET	Positron Emission Tomography
PHF	Paired helical filaments
PS1	Presenilin 1
PS2	Presenilin 2
RNA	Ribonucleic acid
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SSCP	Single-Strand Conformation Polymorphism
TBE	Tris-Boric acid-EDTA buffer
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-Tetramethylethylenediamine
Thr	Threonine
TM	Transmembrane domain
U	Uracil
UTR	Untranslated Region

UV	Ultraviolet
Val	Valine

1. INTRODUCTION

1.1. Alzheimer's Disease

Alzheimer's Disease (AD) was first described by the German neurologist Alois Alzheimer in 1907 in the brain tissue of a woman who had died of an unusual mental illness (Parihar, 2004). AD is the most common cause of dementia in the old population and the fourth leading cause of death in developed countries (Cruts *et al*. 1998). The disease is clinically characterized by a progressive decline in cognitive functions. Most frequent symptoms of AD are impairment in formation of short-term memory, and deficits in language and attention problems. Personality change, deterioration of visuospatial skills, anxiety and depression are common during the course of the disease. Patients eventually lose the ability to become self-supportive and become bedridden. The survival of the disease is 8-10 years, and death usually occurs due to complications which result from immobility and malnutrition (Huber *et al.*, 2006).

In most AD cases, the first symptoms start after the age of 60 years (late-onset or senile AD); however there are cases that the disease onsets earlier in life (early-onset or presenile AD). Both senile and presenile forms of the disease share similar clinical and pathological findings whereas the presenile form, which represents < per cent of all AD cases, shows more rapid clinical progression (Cruts *et al.* 1998). Late-onset Alzheimer's Disease (LOAD) represents the vast majority of all AD cases (Bertram and Tanzi, 2005).

There are environmental factors that may either increase or decrease the risk of AD. Age, smoking, head trauma, depression, lower educational level, atherosclerosis, thyroid disease, exposure to aluminum increases AD risk whereas hormone replacement therapy, alcohol, diet and the use of anti-inflammatory drugs decrease the risk of AD (Sleegers and van Dujin, 2001).

Besides environmental conditions, genetic factors also play a role in AD pathogenesis. To date, more than 160 mutations in three genes have been reported to cause early-onset familial Alzheimer's Disease (EOFAD). These include the *β-Amyloid*

Precursor Protein (*APP*) on chromosome 21, *Presenilin1* (*PS1*) on chromosome 14, and *Presenilin2* (*PS2*) on chromosome 1 (Bertram and Tanzi, 2005). Mutations in the *PS1* gene account for 18-55 per cent of early-onset familial AD cases (Zekanowski *et al.,* 2003).

There are two sets of clinical criteria for the diagnosis of AD. The first is described in the Diagnostic and Statistical Manual-IV (DSM-IV). In order to meet the DSM-IV criteria, memory impairment must be accompanied by deficits in at least one other cognitive domain including visuospatial, language or executive function (Harciarek and Jodzio, 2005). Additionally, cognitive impairment symptoms should be present for at least 6 months and must be severe enough to have an impact on social, occupational, or other daily functions.

The second frequently used criteria for AD was developed by the National Institute of Neurologic and Communicative Disorders and Stroke and the AD and Related Disorders Association Work Group (NINCDS-ADRDA) (Mc Khann *et al.,* 1984). According to this criteria, there must be impairment in at least two areas of cognition, onset between age 40 and 90, and other causes of dementia must be excluded. However, even in the presence of these criteria, the neuropathological examination may not be always consistent with AD, and using the DSM-IV criteria together with NINCDS-ADRDA shows 85-95 per cent of accuracy in diagnosis (Mc Khann *et al.,* 1984). Therefore, postmortem examination of the brain is still necessary to confirm a diagnosis of definite AD.

The major neuropathological characteristics of AD are diffuse loss of neurons, abundant amounts of neurofibrillary tangles and β-amyloid (Aβ) deposited in the form of senile plaques (Tanzi, 1999).

The disease affects up to 15 per cent of people over 65, and nearly half of people over 85 years of age. Since selective neuronal and synaptic loss in the hippocampus and cerebral cortex correlates with clinical symptoms, investigation of the mechanisms that cause neuronal death should lead to a greater understanding of the underlying pathophysiology of the disease and unveil potential therapeutic opportunities (Zhu *et al.,* 2004).

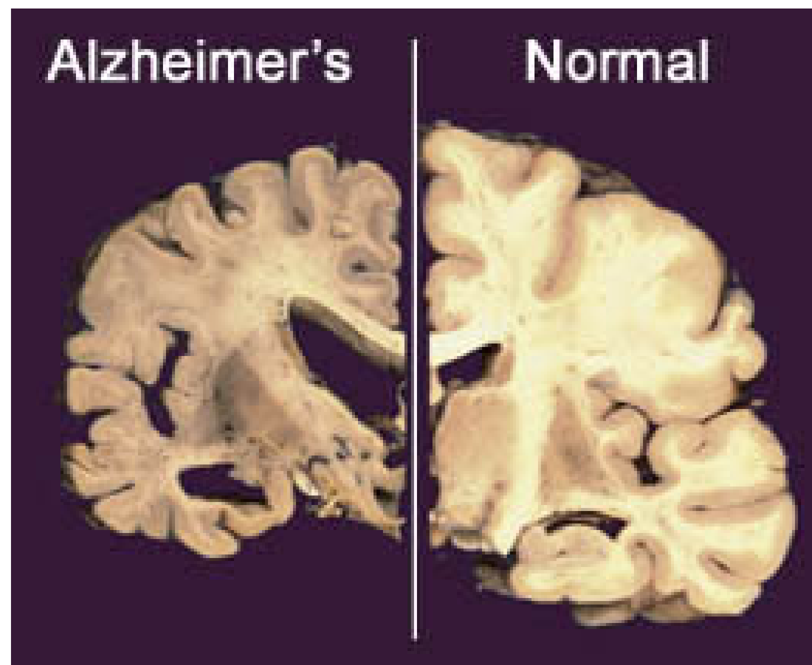

Figure 1.1. Comparison of normal brain (right) with the AD brain (left). Atrophy, and shrinkage of hippocampus is apparent in the AD brain (http://w3.uokhsc.edu/pathology/deptlabs/diagnostic_center_for_alzheimer.htm)

1.1.1. Molecular Pathology of AD

1.1.1.1. Beta Amyloid Plaques. Beta amyloid peptide (Aβ) was first sequenced from the meningeal blood vessels of AD patients and individuals with Down's Syndrome in 1984. A year later, the same peptide was found to be the primary component of the senile plaques found in the brains of AD patients (Masters *et al.*, 1985). The term amyloid was first used by Virchow in 1854, derived from amylose or amylon and means 'starchlike'. The general nature of carbohydrate, present in amyloid, was known to be glycosaminoglycans (Hardy and Selkoe, 2002).

Aβ is a four kDa protein, derived from the proteolysis of membrane bound beta-amyloid presursor protein (APP) which liberates the Aβ peptide via the amyloidogenic pathway. Although Aβ aggregation in the form of senile plaques is detected in AD patients' brain, they are also observed in brains of aged, nondemented individuals. The level of Aβ deposition increases in an age-dependent manner (Morishima-Kawashima and Ihara, 2002).

To date, two subclasses of Aβ plaques have been identified as non-fibrillized (diffuse) and fibrillized (classical) plaques. Presence of diffuse plaques do not appear to affect the structure and function of the neuronal tissue, whereas the classical plaques are shown to be associated with brain damage (Wisniewski *et al.*, 1996).

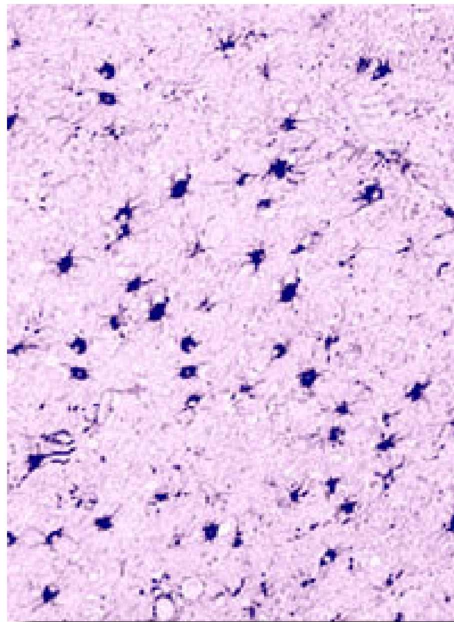

Figure 1.2. Section of an AD brain showing amyloid plaques (http://webs.wichita.edu/mschneegurt/biol103/lecture16/amyloid_plaques.jpg)

1.1.1.2. Neurofibrillary Tangles. The second histopathological hallmark of AD are the neurofibrillary tangles (NFT), that are found in cell bodies, apical dendrites, distal dendrites and in the abnormal neuritis, that are associated with some β-amyloid plaques (neuritic plaques) (Götz *et al.*, 2004). Visualization by electron microscopy has shown that the primary components of NFTs are accumulations of paired helical filaments which consist of the microtubule-associated protein *Tau* (MAPT) that has been phosphorylated abnormally (Grundke-Iqbal *et al.*,1986).

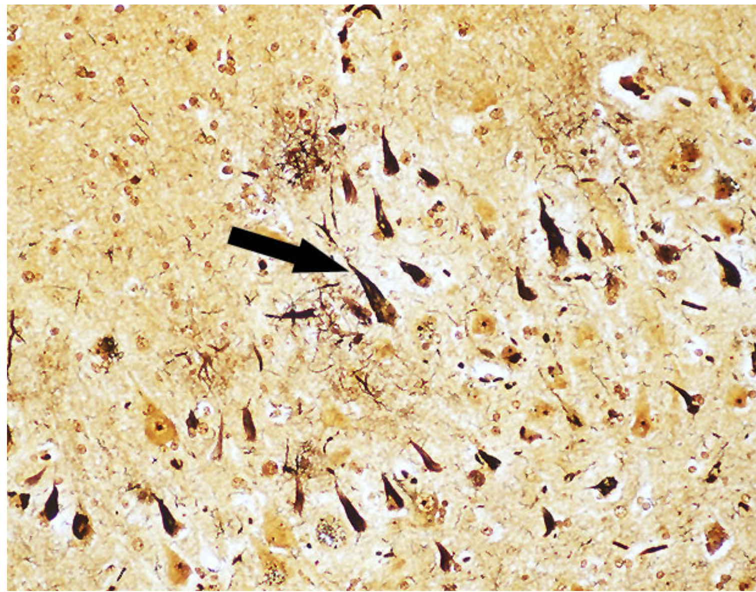

Figure 1.3. Section of the temporal lobe of an AD patient showing neurofibrillary tangles (http://w3.uokhsc.edu/pathology/deptlabs/Alzheimer/Images/p10b.jpg)

Physiological function of *Tau* is the assembly and stabilization of microtubules. It is also thought that *Tau* plays important roles in signal transduction, organization of the actin cytoskeleton, intracellular vesicle transport, and anchoring of phosphatases and kinases (Götz *et al.*, 2004).

Tau is a microtubule (MT)-associated protein of 55 kDa weight and is expressed abundantly in the brain (Morishima-Kawashima, 2002).

In adult human brain, six *Tau* isoforms are produced by the alternative mRNA splicing of exons 2, 3. and 10 of the *Tau* gene, localized on chromosome 17q. They differ by the presence or absence of one or two short inserts in the aminoterminal half, and have either three or four microtubule binding repeats in the carboxy-terminal portion (3R and 4R) (Goedert *et al.*, 1992).

The splicing out of the second 31-residue repeat, encoded by exon 10, gives rise to three-repeat isoforms. Embryonic brains exclusively express three-repeat Tau, which could easily destabilize MTs and affect the ability of neurons to extend or retract their processes (Lee *et al.,* 1991).

In the course of the AD, possibly because of hyperphosphorylation, stabilization of the microtubules is impaired due to reduced ability of Tau to bind microtubules. Phosphorylation results in the dissociation of microtubules (Parihar, 2004).

Following the separation from microtubules, Tau accumulates in the neuronal perikarya especially in dendrites, as paired helical filaments (PHFs), the unit fibrils of neurofibrillary tangles (Morishima-Kawashima *et al.,* 2002).

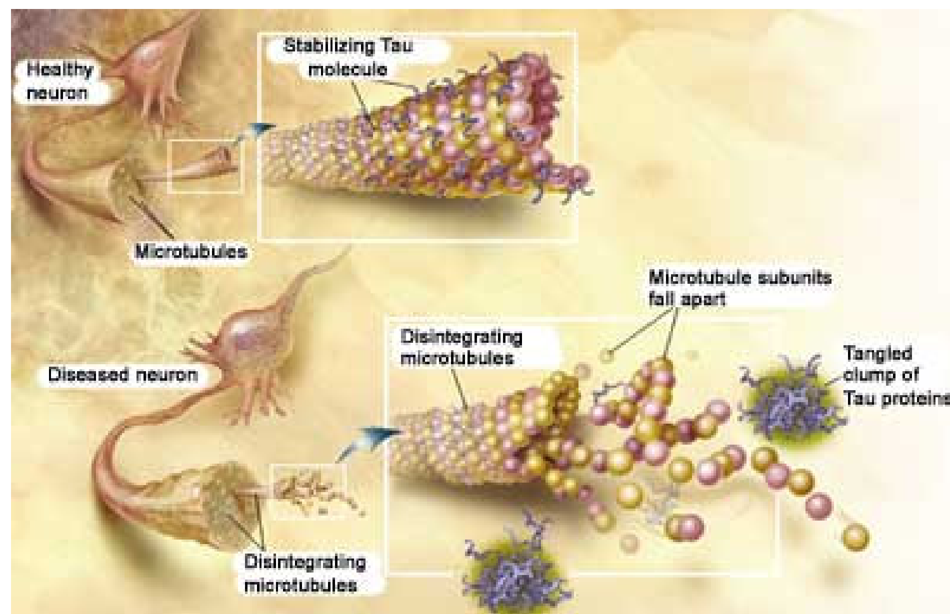

Figure 1.4. Disintegration of microtubules due to the abnormal phosphorylation of *Tau* and process of *Tau* accumulation in the brain (http://www.alzheimers.org/.../ images/TANGLES_LOW.JPG)

More than 25 phosphorylation sites have been identified in PHF-Tau purified from AD brains. Most of the phosphorylation sites are clustered in the flanking region of the MT-binding domain. According to the motifs for protein kinases, multiple protein kinases, including GSK3β, cdk5, MAPK, and PKA, appear to be involved in hyperphosphorylation of Tau. This indicates that multiple phosphorylation cascades may be activated within tanglebearing neurons (Morishima- Kawashima *et al.,* 1995).

In contrast to senile plaques, neurofibrillary tangle formation is a late event and is observed in brains affected by various neurodegenerative diseases as well as AD. Such

diseases that present with intracellular *Tau* inclusions are called *Tauopathies.* It is known for individuals with AD that the formation of NFTs are accompanied by neuronal loss, and the abundance of tangles correlates well with the extent of neuronal loss on the same area. Moreover, the abundance of Tau inclusions or the extent of neuronal loss also correlates well with the degree of dementia. However, some studies on AD brains revealed that the numbers of lost neurons might greatly exceed those of neurofibrillary tangles, indicating that neuronal loss may occur without neurofibrillary tangles. Thus, neuronal loss might not be a consequence of tangle formation and might proceed rather independently. (Poorkaj *et al.,* 1998; Hutton *et al.,* 1998).

1.1.2. Molecular Genetics of Alzheimer's Disease

Almost 100 candidate AD genes were analyzed to date, but only three of them have been proven to play a direct role in AD pathogenesis. These are the *β-Amyloid Precursor Protein* Gene (*APP*), the *Presenilin1* Gene (*PS1*) and the *Presenilin2* Gene (*PS2*); all result in early-onset Alzheimer's Disease (EOAD) when mutated (Bertram and Tanzi, 2004).

While EOFAD mutations in different genes are transmitted in an autosomal dominant fashion, LOAD cases are without obvious familial segregation. Although AD causing mutations occur in three different genes located on three different chromosomes, they all share a common biochemical pathway: Altered production of Aβ, leading to an increase in the ratio of Aβ42, which eventually results in neuronal cell death and dementia (Bertram and Tanzi, 2005).

1.1.2.1. The β-Amyloid Precursor Protein Gene. Intensive molecular and cell biological studies on AD started with the cloning of cDNA encoding β-amyloid precursor protein (APP) in 1987 (Morishima-Kawashima and Ihara, 2002).

- *Structure of the APP Protein*: APP is a member of an evolutionarily conserved family of type I membrane proteins. (Chen and Tang, 2006). APP has a large hydrophilic aminoterminal extracellular domain, a single hydrophobic putative transmembrane domain and a small carboxy-terminal cytoplasmic domain. These domains mature in the endoplasmic reticulum and Golgi apparatus and exhibit post-translational

modifications, including phosphorylation, glycosylation and sulfation (Parihar and Hemnani, 2004).

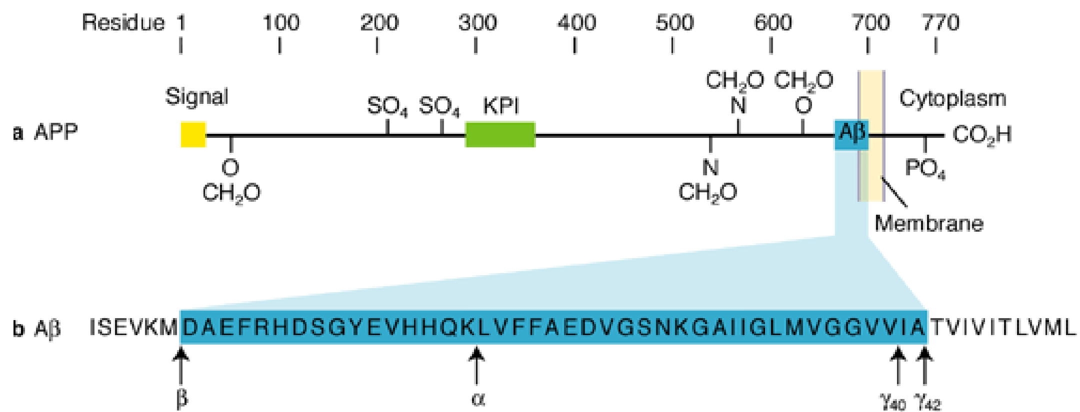

Figure 1.5. The cleavage of APP by α, β and γ secretases to give β-amyloid (http://www-ermm.cbcu.cam.ac.uk/fig001dss.gif)

Catabolic products of APP, which are called the carboxy-terminal fragments (CTF) or stubs, result from the activity of different proteases, named secretases. To date, three different secretase activities have been described; α, β and γ. APP is first cleaved by either the α– or β-secretase activity to form the C83 or C99 fragments, respectively. α-secretase cleave APP in the middle of the Aβ sequence, and this cleavage results in the generation of the Aβ17–40 and Aβ17–42 peptides and another CTF, C83, and prevents the production of amyloidogenic Aβ.

An alternative cleavage by β-secretase occurs at position 11 of Aβ and produces a soluble form of APP and a membrane-bound CTF (CTF, or C99). The subsequent cleavage of C99 by γ-secretase releases Aβ1–40 and Aβ1–42 (Brunkan and Goate, 2005; Irie K. *et al.*, 2005). Aβ40 ends with a Valine at position 40, whereas Aβ42 has two additional hydrophobic residues Isoleucin and Alanin. As a result of this, Aβ 42 is more hydrophobic and has a higher aggregation potential (Morishima-Kawashima and Ihara, 2002).

- *Function of the APP Protein*: The most well known property of APP is being the source of Aβ, found in plaques in AD brain. (Parihar and Hemnani, 2004). Despite plenty of studies on the cell-biological roles of APP, the real functions of APP have

remained unclear. Recent research provided evidence of a novel function of APP as a membrane cargo receptor for kinesin-I, a microtubule motor protein. An axonal membrane compartment, containing APP, γ-secretase, PS1, the neurotrophin receptor TrkA, GAP43, and synapsin I, was identified in a mouse sciatic nerve and cortex. The fast anterograde axonal transport of this compartment was shown to be mediated by APP and kinesin-I. Moreover, proteolytic processing of APP was found to occur in this axonal compartment, both in vitro and in vivo, leading to the production of Aβ, and liberating kinesin-I from the membrane. These findings suggest that APP processing (γ-secretase mediated cleavage) might serve as a cellular mechanism to detach the vesicle from the motor protein once it has arrived at the nerve terminal.

It was demonstrated, and generally accepted that Aβ is a normal product of cellular metabolism. Whether this physiological generation of Aβ is directly linked to axonal transport or not, and whether APP dependent axonal transport of vesicles is actually disturbed in AD or not, should be elucidated in future (Uemura K. *et al.*, 2004).

- *Mutations on the APP Gene and Their Contribution to AD*: 25 pathological mutations in APP have been identified to date (http://www.molgen.ua.ac.be/ADMutations). Most of these are near the proteolytic cleavage sites involved in Aβ production (Spires and Hyman, 2005).The AD causing APP mutations lead to the disease with an onset age from 45 to 60 years (Hardy, 1997).

Mutations of APP are associated with familial AD by increasing the accumulation of Aβ in senile plaques, dystrophic neurites, and synaptic terminals in the AD brain (Spires and Hyman, 2005).

Transgenic co-expression of mutant forms of presenilins and APP showed an increase in the plaque formation and memory impairment (Götz *et al.*, 2004).

1.1.2.2. The Presenilin Genes. The *Presenilin1* gene (OMIM # 104311) is located on cromosome 14 at 14q24.3 and the *Presenilin2* gene (OMIM # 600759) is located on chromosome 1 at 1q42.1. (Cruts *et al.,* 1998) They code the Presenilin proteins PS1 and PS2 which are integral membrane proteins of 467 and 448 aminoacids. They have six to

nine domains with the cytoplasmic N and C termini, and one large hydrophilic loop (HL). The Presenilins show a high degree of homology to each other especially in the transmembrane domains (Hardy, 1997).

The Presenilin1 Gene

- *Structure of the PS1 Gene:* The *PS1* gene was initially discovered by genetic linkage studies in the AD families that inherit the disease in an autosomal dominant manner (Sherrington, 1995). It extends for approximately 75 kb and comprises 12 exons. However, the open reading frame spans only exons 3-12, approximately 24 kb, encoding a protein of 467 amino acids. In the *PS1* gene, exons 1A and 1B represent alternate transcriptional start sites (Dewji, 2005)

- Structure of the Presenilin1 Protein: The structure of the presenilin proteins is proposed to contain six to eight transmembrane domains (TM) with both amino and carboxy terminus as well as the large hydrophilic loop following the sixth transmembrane domain located facing the cytoplasm. Transmembrane domains display the greatest conservation between PS1 and PS2 (Mercken, 1996).

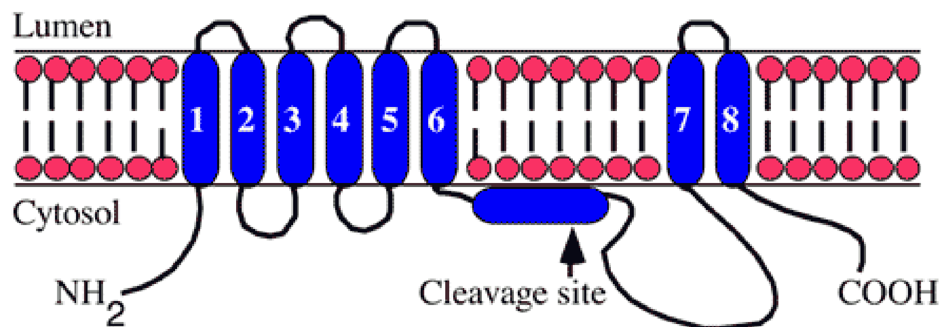

Figure 1.6. Putative 8 transmembrane domains of presenilin proteins (http://ccr.cancer.gov/staff/images/1372_Fortini_179.gif)

The major site of proteolytic cleavage in PS1 has been localized to residue 292 in exon 9, but, cleavage at other adjacent minor positions (291–299) is also observed that generate a heterogeneous population of amino terminal (NTF) and carboxy terminal fragments (CTF) (Thinakaran, 1996). PS1 undergoes endoproteolytic processing within the

cytoplasmic loop connecting putative transmembrane segments 6 and 7, to generate a stable 27 to 28 kDa N-terminal fragment (NTF) and a 16 to 17 kDa C-terminal fragment (CTF). This endoproteolytic process is highly conserved and a processing event that regulates the accumulation of proteins (Thinakaran, 2004).

PS1 is ubiquitously expressed in peripheral tissue and in the nervous system. Subcellular localization of PS1 in neurons has been investigated using biochemical methods, immunostaining and immunoelectron microscopy (Thinakaran, 2004).

Besides showing 67 per cent homology to each other (Dewji NN., 2005), both presenilins are ~50 per cent homologous to Sel-12 which is involved in cell signaling by Notch-based receptors (Janicki and Monteiro, 1997).

- *Functions of the Presenilin Proteins:* Functions of the Presenilin proteins are unclear.

Initially, PS1 was thought to be the γ-secretase, but subsequently it was shown that γ-secretase is a complex including presenilin, nicastrin and other molecules such as PEN-2 and APH-1 (Kimberly WT *et al.,* 2003). Expression of the *Presenilin1* has been shown at the cell surface membrane, in the endoplasmic reticulum and in the Golgi apparatus by various studies with transfected cells (De Strooper, 1997). In addition to these, PS1-derived fragments in neurons were also found in small synaptic vesicles, synaptic plasma membranes, synaptic adhesion sites and neurite growth cone membranes. These findings indicate that PS1 may regulate neuronal differentiation, development and/or synaptic function (Thinakaran and Parent, 2004).

Recent genetic, biochemical and pharmacological evidence shows that, PS1 functions as a multiprotein complex, coprised of at least three other transmembrane poteins namely nicastrin, Aph-1 and Pen-2 (Thinakaran and Parent, 2004). PS1 endoproteolysis and accumulation of fragments are regulated by the presence of these three proteins (Kimberly *et al.,* 2003). All three proteins were first identified by genetic screens performed in *Caenorhabditis elegans* and were shown to exhibit γ-secretase activity. Each of these proteins appears to be dependent on each other for biogenesis, maturation and stability. The highly glycosylated membrane protein nicastrin was shown to be unmature and leave the endoplasmic reticulum in cells lacking PS1 expression (Leem, 2002).

Presenilins, like APP, are processed by being cleaved in exon 9, yielding a ~25 kDa N-terminus fragment and a ~19 kDa C-terminus fragment. It is not at all clear, whether the holoprotein or either or both of the protein fragments are physiologically or pathologically important (Mercken, *et al.*, 1996).

The finding that presenilins interact with Bcl-XL, a member of the anti-apoptotic Bcl-2 family of proteins, offers a potential mechanism that presenilins might regulate apoptosis (Passer *et al.*, 1999).

PS1 also has impacts on Notch activity, which is essential for the cell fate decisions in the embriyogenesis, and which plays a fundamental role in the adult, in the regulation of neurite outgrowth. The inhibitory effect of Notch signaling on neurite outgrowth is found to be reduced in *PS1 -/-* neurons suggesting that PS1 facilitates Notch1 function in post-mitotic mammalian neurons. Pharmacological inhibition of PS1/γ-secretase activity decreases Notch dependent neurite outgrowth in cultured human NT2N cells (Thinakaran and Parent, 2004).

Furthermore, a model was proposed in 1996, that the formation of the Aβ from APP requires a specific cell to cell interaction in the brain, that is mediated by the direct and specific interaction of APP on one cell surface with either PS1 or PS2 on the other, as the first step in the eventual production of Aβ (Marambaud, 2002). In the same year, Dewji et al. presented the first evidence for a specific interaction in cell culture between cell surface APP and either PS1 or PS2 on the DAMI cells. on a second cell. They demonstrated that when APP-transfected human DAMI cells were mixed, under appropriate conditions, with either PS1 or PS2 transfected DAMI cells, cell-cell aggregation was observed under light microscopy that did not occur if vector-only transfected DAMI cells were used instead of either transfected cell (Dewji *et al.,* 1996).

In *PS1/PS2* double knock-out cells, Aβ secretion into the medium is completely inhibited. There is now strong, but contesting evidence, that supports the claim that PS is itself the protein with γ-secretase activity, when it is the part of a complex containing nicastrin, Pen-2 and Aph-1. Involvement of this complex in the intramembranous cleavage

of several type I transmembrane proteins, including APP, Notch and ErbB4 has been proven (Sisodia *et al.*, 2001).

- *Mutations on the PS1 Gene and Their Contribution to Alzheimer's Disease:* 158 mutations were reported on the *PS1* gene to date (http://www.molgen.ua.ac.be/ADMutations).

The majority of *PS1* mutations are missense mutations although deletions and insertions can be seen. Missense mutations in the *PS* genes are a major cause of EOFAD. Those missense mutations causing amino acid substitutions result in a relative increase in the ratio of the Aβ42 to Aβ40. When transfected into cell lines, most *PS1* mutations result in the increased production of the Aβ42 (Larner and Doran, 2006). Cell culture studies showed that mutations in *PS* affect γ-secretase complex generation and suggest that clinical mutations could cause loss of function, not only by interfering with the catalytic efficiency of the protease, but also by modifying the docking of substrates (Bentahir *et al.,* 2006).

Very early onset of the disease has been noted with the mutations: L85P, P117L, P117S, insF1, L166P (seizures in adolescence, cognitive decline in 20s), S169L, M233L, M233V, L235P, Y256S, V272A, A434C, P436Q, and possibly G206V (behavioral and psychiatric features more prominent). Several mutations, associated with the very early onset of the disease suggest that these mutations alter the conformation of the presenilin protein by substituting hydrophilic and hydrophobic amino acids. Although there are some exceptions: A79V, M139V, I143F, H163R, H163Y, A231V, L271V, E273A, and possibly C410Y ; mutations of *PS1* show almost complete penetrance by the age of 60 years. The factors contributing to the reduced penetrance are yet unclear. Direct correlation between mutation site and phenotype could not be seen thus far, suggesting that the site of the mutation on *PS1* is unimportant; most of the mutations act as toxic gain of function mutations affecting γ-secretase function (Larner and Doran, 2006).

While the mutations are found throughout the PS proteins, they generally occur at residues conserved between the PS, and which are also conserved in *sel-12,* the *C. elegans* PS homologue. In relation to this, they occur largely in transmembrane domains.

The larger proportion of familial AD patients (50–70 per cent of all cases 50 families) have mutations within the *PS1* gene (Janicki and Monteiro, 2006). Most *PS1* mutations reported so far had onset ages between 25 and 55 years (Cruts *et al*., 1998). The major cluster of mutations is in exon 8. This mutation cluster is of interest both because it is close to the cleavage site, and because in both PS1 and PS2 there are minor isoforms in which this exon is skipped. This suggests that this exon might be a functional domain (Cruts *et al.* 1996).

The proximity of the second cluster to the site of cleavage in the presenilin protein may be significant to the effect of these mutations on the function of PS1. The mutations in TM2 in PS1 line up on one side of the α-helix, suggesting that this helical face is important, probably in interacting with other TM domains or other TM proteins (Crook *et al.,* 1997).

Mutations in both *PS1* and *PS2* are associated with increased production of Aβ42, the amyloidogenic form of Aβ that is deposited selectively and early in AD. A similar increase is not found in the level of Aβ40 (Mann *et al.*, 1997).

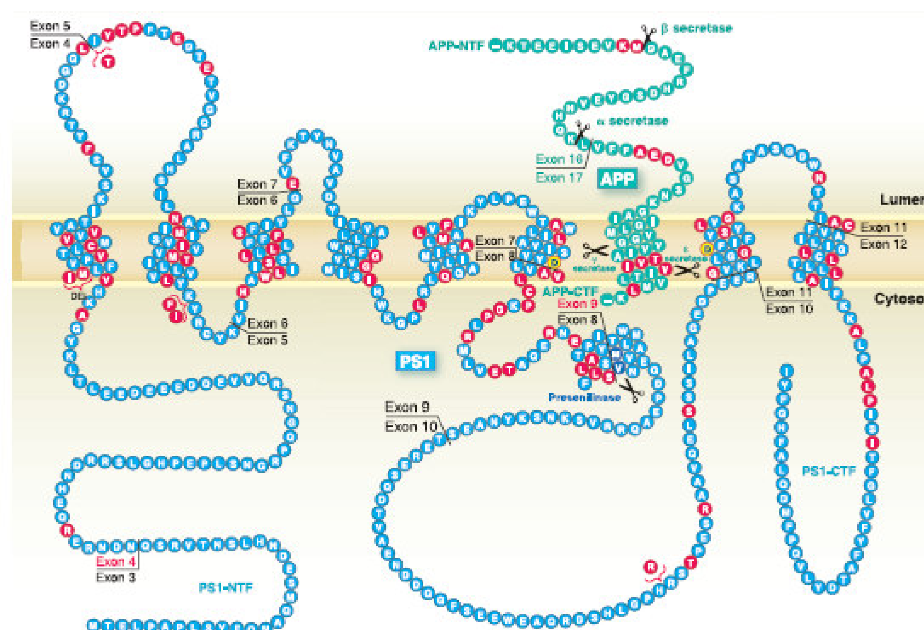

Figure 1.7. Entire amino acid sequence of PS1 (blue) and a portion of the COOH-terminal sequence of APP (green) is shown. (Hardy and Selkoe, 2002)

The Presenilin2 Gene

- *Structure of the PS2 Gene:* The PS2 gene was identified because of its close homology (67 per cent) to PS1 (Levy-Lahad *et al.,* 1995). It has 10 protein coding and two non-coding exons. Exon 8 of *PS2* is alternatively spliced as in *PS1*. The *PS2* promoter lacks consensus TATA and CAAT boxes, but contains GC rich sequence regions upstream of two transcriptional start sites.

The equivalent positions of intron-exon boundaries in the coding sequence of *PS1* and *PS2* are nearly identical, consistent with the idea that they are derived from a common ancestral gene that underwent gene duplication (Prihar *et al.*, 1996).

- *Structure of the Presenilin 2 Protein: PS2* mRNAs are 2.3 and 2.6 kb in size (Hutton and Hardy, 1997). The full-length PS2 protein (53–55 kDa) is proteolytically cleaved to yield a 35 kDa N-terminal fragment and a 20 kDa C-terminal fragment (Parihar and Hemnani, 2004).

Although expression of *PS1* appears to be at approximately equivalent levels in all tissues, the highest levels of *PS2* expression is observed in heart, pancreas and brain by Northern blot analysis (Hutton and Hardy, 1997).

- *Mutations on the PS2 Gene and Their Contribution to Alzheimer's Disease:* Initial linkage studies indicated that *PS2* mutations are less frequent than *PS1* mutations. 11 mutations were detected on the *PS2* gene up to date (http://www.molgen.ua.ac.be/ ADMutations). Furthermore, in contrast to *PS1* families, age of onset is generally later (45–88 years) than that observed for most *PS1* mutations (25–65 years) and is highly variable even among affected members of the same family (Sherrington R. *et al.*, 1996, Bird *et al.*, 1996).

According to the common opinion that mutations in *PS1* and *PS2* could cause AD by the deposition of Aβ, transfection experiments were done in cultured cells. In the framework of these studies, wild-type *PS2* cDNAs and cDNAs with the N141I (Arg141Ile) mutation found in EOFAD patients from Volga German families were used to

examine the metabolism of PS2, as well as the levels and the C-terminal properties of Aβ secreted from cultured cells.

N141I mutant *PS2* increased the levels of secreted Aβ42 by 1.5- to 2-fold compared to those with wild-type *PS2*, whereas the secretion of Aβ40 was decreased by 20 to 50 per cent. The mutant form of *PS2* resulted in a 1.5- to 3.8-fold increase in the percentage of total Aβ [Aβ40+Aβ42] (Tomita *et al.*, 1997).

The N141I mutation associated with AD also caused an increase in apoptosis in cells overexpressing the mutant protein. Deletion studies have indicated that retention of the NH2-terminal *PS2* sequences are necessary for apoptosis (Janicki and Monteiro, 2006).

1.2. Frontotemporal Dementia (FTD)

1.2.1. Molecular Pathology of Frontotemporal Dementia

Frontotemporal dementia is a degenerative brain disease that is the second most common form of early-onset dementia (Stanford *et al.*, 2003) and has overlapping features with AD (Dermaut *et al.*, 2005). Clinical symptoms of FTD include behavioral changes, loss of frontal executive functions, language deficit and hyperorality. These changes are followed by cognitive decline leading to profound dementia (Tolnay and Probst, 2003). However; there is a clinical heterogeneity described between and within the families with FTD. Disease sometimes may be accompanied by Parkinsonism and amyotrophy (Sergeant *et al.*, 2005).

Neuropathological examination of the brains of FTD patients reveals atrophy of the frontal and temporal lobes, a severe neuronal cell loss, a grey and white matter gliosis, and abundant intraneuronal Tau inclusions. Presence of amyloid aggregation is not usually established (Spillantini *et al.*, 1998; Sergeant *et al.*, 2005).

1.2.2. Molecular Genetics of FTD

Frontotemporal dementias occur in both familial and sporadic forms. Familial form of the disease has been related to mutations on the Tau gene. FTDP-17 is inherited in an autosomal dominant manner (Ingram and Spillantini, 2002). The onset age of FTD is highly variable and ranges from the early 20s to late 70s, with a mean age of 50 years (Rademakers *et al.*, 2004).

1.2.2.1 The Tau Gene.

- *Structure of the Tau Gene:*

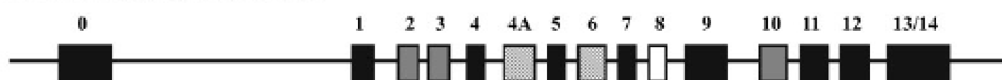

Figure 1.8. Structure of the *Tau* gene showing exons

The human Tau gene is on the chromosome 17q21 and contains 16 exons (Andreadis *et al.,* 1992). On the promoter region and the exon 9, there are two CpG islands. The three SP1-binding sites are suggested to control neuronal specific expression of Tau by initiating transcription (Sergeant *et al.*, 2005).

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- *Structure of the Tau Protein:* The *Tau* protein was first isolated as an MT-binding protein that induced the assembly of MTs from purified tubulin (Rademakers *et al.*, 2004). The *Tau* primary transcript contains 16 exons. However, three of them (exons 4A, 6 and 8) are skipped in human brain. They are specific to peripheral *Tau* proteins. Exon 4A is found in bovine, human and rodent peripheral tissues with a high degree of homology. Exon1 is non-coding. Exons1, 4, 5, 7, 9, 11, 12 and 13 are constitutive exons. Exons2, 3 and 10 are alternatively spliced and are adult brain-specific (Rademakers *et al.,* 2004).

Exon3 never appears independently of exon2. Thus, alternative splicing of these three exons allows for six combinations which range from 352 to 441 amino acids with

molecular weights ranging from 45 to 65 kDa: [2(-),3(-)10(-); 2(+)3(-)10(-); 2(+)3(+)10(-); 2(-)3(-)10(+); 2(+)3(-)10(+); 2(+)3(+)10(+)] (Sergeant *et al.*, 2005).

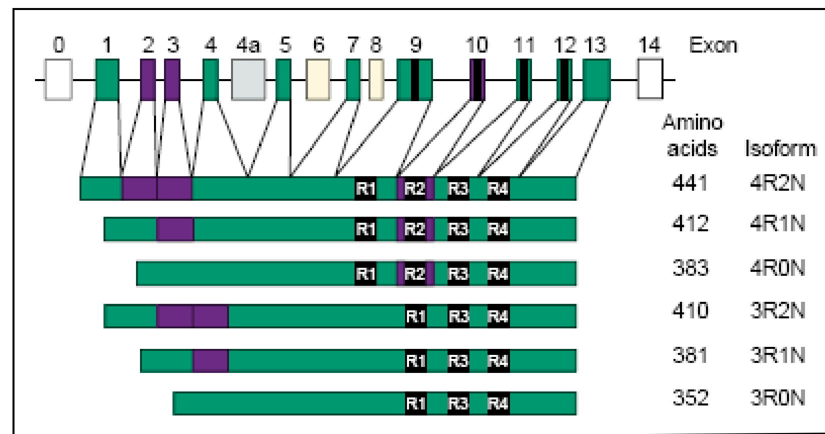

Figure 1.9. The *Tau* primary transcript, gives rise to six mRNAs in the human brain. The *Tau* isoforms differ from each other by the presence of either three (3R) or four repeat regions (4R) in the carboxy-terminal (C-terminal) part of the molecule and the absence or presence of one or two inserts (29 or 58 amino acids) in the amino-terminal (N-terminal) part

It is thought that each of these isoforms have specific physiological roles since they are differentially expressed during development (Munoz *et al.,* 2003). Three of these isoforms that contain three repeats (3R-Exon 10 missing) are thought to be involved in binding to microtubules (Andreadis *et al.*, 1992).

In the fetal brain, only the transcript encoding the shortest *Tau* isoform with three repeats is expressed in contrast to the adult brain where, all six isoforms are expressed suggesting that *Tau* expression is developmentally regulated (Munoz *et al.,* 2003; Ingram *et al.,* 2002).

Thus far, exon8 has not been observed in human MAPT transcripts. Expression studies in different species identified exon8 containing transcripts in Rhesus monkey and in small amounts in cow (Rademakers *et al.,* 2004).

Phosphorylation of the *Tau* protein is developmentally regulated and fetal *Tau* is more phosphorylated than adult brain *Tau* (Rademakers *et al.*, 2004). There are ~80

putative Ser or Thr phosphorylation sites on the longest brain *Tau* isoform (441 amino acids length) (Sergeant *et al.*, 2005).

- *Mutations on the Tau Gene and Their Contibutions to Frontotemporal Dementia:* It was shown that mutations in the gene encoding the microtubule associated protein Tau (*MAPT*) cause autosomal dominant forms of frontotemporal dementia (FTD). The first mutations in *MAPT* causing autosomal dominant FTD were identified in 1998 (Poorkaj *et al.,* 1998).

Fifty eight different causative *MAPT* mutations have now been reported. Mutations on the *MAPT* gene include missense, silent and intronic variations and two single codon deletions (http://www.molgen.ua.ac.be/ADMutations). Tau mutations always segregate with the pathology and are not found in the control subjects, suggesting their pathogenic role.

Depending on their functional effects, mutations on Tau proteins may be divided into two groups: the mutations affecting the alternative splicing of exon 10, and leading to changes in the proportion of 4R and 3R Tau isoforms, and the mutations modifying Tau interactions with microtubules.

The first group includes intronic mutations and some missense mutations. Intronic mutations are on a stem loop structure in the 5' splice site of exon 10 that stabilizes this region of the pre-mRNA. Sequence analysis of this splicing region in different animals indicates that the lack of the stem loop structure is associated with an increase in *Tau* mRNAs containing exon 10 and thus the 4R *Tau* isoform. Some missense mutations (N279K and S305N) also modify the splicing of exon 10. For instance, the change in nucleotide for N279K and S305N mutations also creates an exon-splicing enhancer sequence. The silent mutation L284L increases the formation of *Tau* mRNAs containing exon 10, presumably by destroying an exon splicing silencing element.

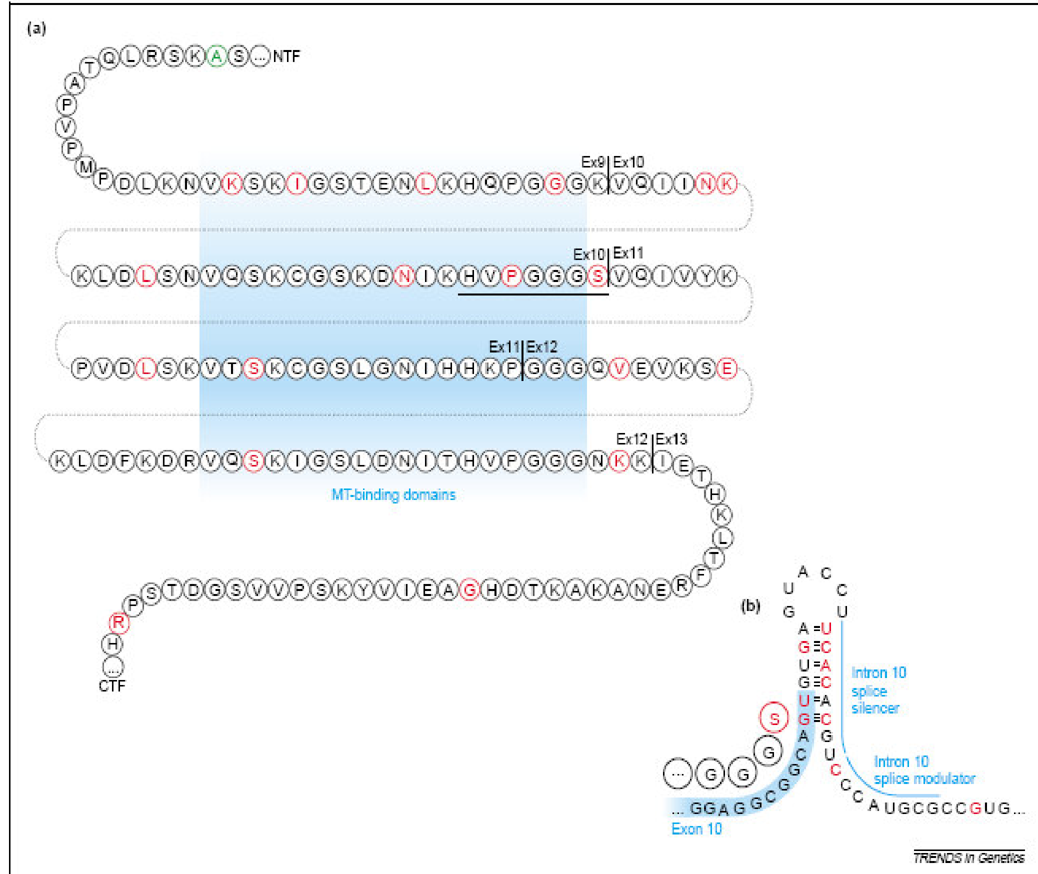

Figure 1.10. *MAPT* mutations. (a) Schematic representation of *MAPT* exons nine to 13 encoding the four microtubule-binding domains and inter-repeat regions of the Tau protein. Each circle represents an amino acid; those with pathogenic missense mutations are shown in red and those with non-pathogenic missense mutations are in green. (b) The 3' end of exon ten and 5' end of intron ten of *MAPT*, showing the pathogenic mutations in this region in red (Dermaut *et al.*, 2005)

The second group of *Tau* mutations found in FTD includes several missense mutations. The effects of mutations G272V, P301L, P301S, V337M and R406W in an in vitro system of microtubule assembly showed that mutated *Tau* isoforms bind microtubules to a lesser extent than wild-type isoforms. They suggest that the mutated isoforms may induce microtubule disassembly.

When missense mutations are located in *Tau* regions common to all isoforms, outside exon 10 (V272G, V337M, G389R, R406W), the six Tau isoforms do not bind properly to

microtubules. These proteins aggregate into PHF and straight filaments similar to those described in AD, and are present in neuronal cells. On the contrary, when missense mutations are located in exon 10 (P301L, P301S), 4R Tau isoforms do not bind to microtubules, and aggregate into twisted ribbon filaments. This type of filamentous inclusions is described in both neurons and glial cells.

Mutations in exon 10, as well as the intron 10 mutations, affect only 4R Tau isoforms or their expression levels (Sergeant *et al.*, 2005).

2. PURPOSE

Alzheimer's Disease and Frontotemporal Dementia are the most common causes of dementia worldwide. Since diagnoses, based on neuropsychological and neuroimaging examinations are not definite, their genetic analyses could be helpful for the complicated differential diagnoses, and for genetic counselling and predictive testing of these disorders.

The aims of this study are:

- Molecular analyses of patients with Alzheimer's Disease and Frontotemporal Dementia

- Evaluation of the contributions of *PS1* and *PS2* genes to Alzheimer's Disease and of *MAPT* gene to Frontotemporal Dementia.

3. MATERIALS

3.1. Human Blood Samples

Blood samples of Alzheimer's Disease patients were provided by Neurology Departments of İstanbul University İstanbul Medical School, İstanbul; Gülhane Military Medical Academy, İstanbul; Akdeniz University Medical School, Antalya; Celal Bayar University Medical School, Manisa, and Marmara University Medical School, İstanbul.

Frontotemporal Dementia patients were referred to our laboratory from Neurology Departments of İstanbul University, İstanbul Medical School and Marmara University Medical School, İstanbul.

3.2. Equipment

Facilities of the Department of Molecular Biology and Genetics at Boğaziçi University (İstanbul, Turkey) were used for this thesis (Table 3.1.).

3.3. Buffers and Solutions

All chemicals and solutions were purchased from Merck (Germany), Sigma (USA and Germany) or AppliChem (Germany), unless stated otherwise in the text (Table 3.2.).

3.4. Fine Chemicals

3.4.1. Enzymes

Taq DNA and GoTaq Polymerases were from Promega, USA.

3.4.2. Primers

Primers used in this thesis are listed on Table 3.3. Primers were synthesized by Iontek, İstanbul.

Table 3.1. Equipment used in this thesis

Equipment	Model / Manufacturer
Autoclave	Model MAC-601, Eyela, Japan
Balance	GM 512-OCE, Sartorius, Germany
Centrifuges	Centrifuge 5415C, Eppendorf, Germany Universal 16R, Hettich, Germany
Deep Freezers (-20°C)	Arçelik 2021D, Turkey
Documentation System	GelDoc Documentation System, BIO-RAD, USA
Electrophoresis Equipments	Horizon 58, Model 200, BRL, USA Thermo Minicell Primo E320 Electrophoretic Gel System, USA
Gel drier	Easy Breeze Drying Frame (Hoefer Scientific Instruments, Germany)
Magnetic Stirrer	Chiltern Hotplate Magnetic Stirrer, HS31, UK
Ovens	EN 400, Nuve, Turkey 56°C, LEEK, UK
Power supplies	EC 1000-90 Thermo, USA EC 250-90 Thermo, USA Model 200, BRL, USA
Refrigerator	4°C Medicool, Sanyo, Japan Arçelik 4250T, Turkey
Shakers	Heidolph, Unimax, 1010
Spectrophotometer	CE 5502 Scanning Double Beam 5000 Series CECIL Elegant Technology, UK
Thermocyclers	Techne Progene, UK Techne Touchgene Gradient, Progene, UK
Vortex	Fisons WhirliMixer, UK
Water Purification	WaTech Water Technologies, Turkey

Table 3.2. Buffers and solutions used in this study

DNA Extraction from Whole Blood

Cell Lysis Buffer	155 mM NH_4Cl 10 mM $KHCO_3$ 1 mM Na_2EDTA (pH 7.4)
Nuclei Lysis Buffer	400mM NaCl 10 mM Tris-HCl (pH 8.0) 2 mM Na_2EDTA (pH 7.4)
Sodiumdodecylsulphate (SDS)	10 per cent SDS (w/v) (pH 7.2)
Proteinase K	20 mg/ml in dH_2O, Promega, USA
Sodium Chloride (NaCl)	5 M saturated stock solution
Ethanol (EtOH)	Absolute ethanol
TE Buffer	20 mM Tris-HCl (pH 8.0) 1 mM Na_2EDTA (pH 8.0)

Agarose Gel Electrophoresis

10X TBE Buffer	1 M Tris-Base 900 mM Boric acid 20 mM Na_2EDTA (pH 8.3)
Ethidium Bromide (EtBr)	10 mg/ml, Sigma, Germany
1 or 2 per cent Agarose Gel	1-2 per cent agarose (w/v) in 0.5X TBE Buffer, containing 0.5 µg/ml Ethidium Bromide
10X Loading Dye	2.5 mg/ml Bromophenol Blue (BP) 1 per cent SDS in glycerol
100 bp DNA Ladder	100 base pair (bp), MBI Fermentas, Lithuania

Polyacrylamide Gel Electrophoresis

Denaturing Loading Dye	0.05 % xylene cyanol 0.4 % mg/ml BPB 94% Formamide
25% Acrylamide	49:1 Acrylamide/Bisacrylamide in dH_2O
10% APS	10% Ammoniumpersulfate in dH_2O
TEMED	N,N,N,N´,-Tetramethyletylenediamine

Silver Staining of Polyacrylamide Gels

Fixation solution	10% Ethanol
Oxidation solution	1% Nitric acid
Silver reaction solution	0.2% w/v Silver nitrate 0.037% w/v Formaldehyde
Developing solution	3% w/v Sodium carbonate 0.037% w/v Formaldehyde 0.002% w/v Sodium thiosulphate
Stopping solution	10% Acetic acid

Table 3.3. Primers (5→3) used in the framework of this thesis

Gene	Exon	Primer	Sequence	Product size (bp)
PS1	1A	S182ex1A-3 S182ex1A-4	TTCTCCCCGCAATCGTTTCTCCAG GCCCATGTCCGCGGTGCCTTCC	297
	1B	S182ex1B-3 S182ex1B-4	AGGAGGGGCGGCCCGTTTCTCGAGC CTCTGCCACCACCGNAGGATC	523
	2	S182ex2-3 S182ex2-4	TGGATGACCTGGTGAAATCCTATT CAGAAAACAAAGCCTCTTGAGGTT	223
	3	S182ex3-1 S182ex3-2	ACAAAGTTCTGTTTTTCTTTCCC CAGCATTTCTCAGAGGTGAGG	247
	4	S182ex4-1 S182ex4-2	CGTTACCTTGATTCTGCTGA GACATGCTGTAAAGAAAAGCC	371
	5	S182ex5-3 S182ex5-4	GATTGGTGAGTTGGGGAAAAGTG ATACCCAACCATAAGAAGAACAGG	335
	6	S182ex6-3 S182ex6-4	GGTTGTGGGACCTGTTAATT TTAATTCTGAAAGACAGACCC	149
	7	S182ex7-1 S182ex7-2	GGAGCCATCACATTATTCTAAA AACAAATTATCAGTCTTGGGTTT	326
	8	S182ex8-1 S182ex8-2	TTACAAGTTTAGCCCATACATTTT TCAAGTTCCCGATAAATTCTAC	215
	9	S182ex9-1 S182ex9-2	TGTGTGTCCAGTGCTTACCTG TGTTAGCTTATAACAGTGACCCTG	188
	10	S182ex10-1 S182ex10-2	CCAGCTAGTTACAATGACAGC TCAAAAAGGTTGATAATGTAGCT	345
	11	S182ex11-1 S182ex11-2	GGTTGAGTAGGGCAGTGATA TTAAAGGGACTGTGTAATCAAAG	275
	12	S182ex12-1 S182ex12-2	GTCTTTCCCATCTTCTCCAC GGGATTCTAACCGCAAATAT	199
PS2	1	PS2ex1-3 PS2ex1-4	TGTTAGCAGCGGTGTTTG TCTGCTCGGAGGGATGGAC	248
	2	PS2ex2-1 PS2ex2-2	CAGGGCCAGGGGGAGGAA AAAAGCAGGTTGGGAGTCAC	303
	3	PS2ex3-1 PS2ex3-2	GTCCTCCACTGCCTTTGTCTCAC CTTCCCTTCTCCCTCCCGCATCAG	328
	4	5PS2ex4 3PS2ex4	AAAAATCCGTGCATTACAT GCTGGTTGTGAGCTGCAGGTACAGTG	395
	5	PS2ex5-1 PS2ex5-2	AGCCTCGAGGAGCAGTCAG GCAGACGGAGAAGCGT	241
	6	5PS2ex6 3PS2ex6	GGTATCAGTCTCAGGATCATGGG TGGGGAAGACTGGAGCTCGATG	265
	7	PS2ex7-1 PS2ex7-2	GTAAAGAGGGCCAGGTTGGG GTGCAGCACTGGGGACGATTT	387
	8	5PS2ex8 3PS2ex8	GGGCAGGCTCTTCTTCAGGG GAAAGCCACGGCCAGGAAG	251
	9	PS2ex9-3 PS2ex9-4	ACCGCCTGAGACGTGAACCCT TCCCTCTGCCCCTCCTGAACT	235
	10	5PS2ex10 3PS2ex10	CTCTGACCAGCTGTTGTTTC AGCCTCCACCCTCTGTCT	249
	11	5PS2ex11 3PS2ex11	TTCCATTCTGTGCACGCCTC ACCTGCCCCCACCACAATG	244
	12	PS2ex12-3 PS2ex12-4	ACACCAGCGGATCACCACGCTCAC TGCCTCCTCCTCACCAAGTAAACA	344

Table 3.3. (Continued) Primers (5→3) used in the framework of this thesis

Gene	**Exon**	**Primer**	**Sequence**	**Product size (bp)**
MAPT	9	MAPTex9	CGAGTCCTGGCTTCACTCC CTTCCAGGCACAGCCATACC	370
	10	MAPTex10	GGTGGCGTGTCACTCATCC GTACGACTCACACCACTTCC	200
	11	MAPTex11	GCTCATTCTCTCTCCTCCTC GCAGTTCCAGCCTCACCAGG	188
	12	MAPTex12	GTCCTGTCATTGTCTTCTTC ACCCACTGGATGCTGCTGAG	437
	13	MAPTex13	CTTTCTCTGGCACTTCATCTC CCTCTCCACAATTATTGACCG	299

4. METHODS

4.1. DNA Extraction from White Blood Using The NaCl Method

Peripheral blood samples were collected from individuals into vacutainer tubes containing EDTA as anticoagulant and stored at $4^{o}C$ until DNA extraction up to seven days. The samples were transferred to Falcon tubes, and 30 ml cold lysis buffer for every 10 ml of blood was added. After shaking the samples very well, they were kept at $4^{o}C$ for 15 minutes for the lysis of the white blood cells. After calibrating for weight, the samples were centrifuged at 5000 rpm for 10 minutes at $4^{o}C$ to collect the nuclei. The supernatant containing the RBC debris was discarded carefully. The nuclear pellet was washed by adding 10 ml lysis buffer and resuspended by vortexing. The samples were again centrifuged at 5000 rpm for 10 minutes at $4^{o}C$. The supernatant was discarded carefully. If the pellet was not white, it was washed with lysis buffer, and the centrifugation steps were repeated. The nuclear pellet was resuspended in 3 ml nuclei lysis buffer by vortexing to lyse the nuclear envelope of white blood cells. Following the addition of 400 µl dH_2O, 50 µl SDS (10 per cent), 30 µl proteinase K (20 mg/ml), the samples were incubated at $37^{o}C$ overnight, or at $56^{o}C$ for three hours, for the degradation of cellular proteins. When the incubation period ended, 5 ml dH_2O and 5 ml of 5 M NaCl were added and the samples were shaken well. This step is required to salt out the proteins. The samples were centrifuged at 5000 rpm at room temperature for 30 minutes. The supernatant was transferred into another Falcon tube, and DNA was precipitated with the addition of two volumes of cold absolute ethanol. The tube was inverted slowly several times. The precipitated DNA was fished out by the tip of a micropipette and transferred into an Eppendorf tube. After waiting for the ethanol to dry, DNA was dissolved in 200-500 µl TE buffer at room temperature (overnight). If there was no visible DNA precipitate after inverting the tube several times, the sample was stored at $70^{o}C$ for two hours. The sample was centrifuged at 14000 rpm for 30 minutes at $18^{o}C$. The supernatant was discarded carefully without losing the pellet. When ethanol dried, the DNA pellet was washed with 100 µl TE buffer.

4.2. Qualitative and Quantitative Analysis of the Extracted DNA

To test DNA quality and quantity, DNA samples were run on a 1 per cent agarose gel. 0.3 g agarose was dissolved in 30 ml 0.5X TBE buffer. After addition of 1.6 µl EtBr (10 mg/ml), the gel was poured into a gel plate with one or two combs inserted in it, and allowed for cooling. Then, the gel was placed in the electrophoresis tank filled with 0.5X TBE buffer, and the combs were taken out. 1µl of DNA was mixed with 5µl of 6X loading dye and applied into the slots using a micropipette. The gel was run for 10 minutes at 150V; the DNA bands were visualized under UV light.

The exact concentration of the DNA was determined spectrophotometrically. A 1:100 dilution of the stock DNA (with dH_2O) was prepared and dH_2O was used as blank. The optical density (OD) of DNA was read at 260 nm using a quartz cuvette. Since 50 µg/ml double stranded DNA has an OD of 1.0 at 260 nm, the following formula was used to calculate the concentration of the stock DNA: Concentration (µg/ml): OD_{260} x 50 µg/ml x Dilution Factor.

4.3. Investigation of Mutations

4.3.1. Presenilin Genes

4.3.1.1. Amplification of PS1 and PS2 Exons by PCR. For the detection of mutations on the *PS* genes, 1µl of genomic DNA was amplified in a 25µl reaction mixture containing 2.5 µl of 10X PCR reaction buffer, 0.2 µl of 25 mM dNTP mix, 0.5 µl of each primer (from 50µM stock solution), 0.2 µl of Taq polymerase (5Units/µl). The final $MgCl_2$ concentration of each reaction was adjusted according to Mg^{+2} concentrations given on Table 3.4. DMSO was used for some of the PCR reactions to inhibit non-specific binding and also production of the primer dimers. The final volume of the PCR mixture was completed to 25 µl with the addition of ultra pure dH_2O.

The amplifications of the *PS1* and *PS2* exons by PCR were carried out using the conditions below:

Initial Denaturation at	: 94 ^{o}C	4'	
Denaturation at	: 94 ^{o}C	1'	X cycles
Annealing at	: X ^{o}C	1'30''	X cycles
Extension at	: 70 ^{o}C	2'	X cycles
Final Extension at	: 72 ^{o}C	6'	

The annealing temperature and number of cycles for each reaction are shown on Table 3.4. The quality and quantity of the PCR products were determined on a two per cent agarose gel.

4.3.1.2. SSCP Analysis by Using Polyacrylamide Gel Electrophoresis. 12.5 per cent polyacrylamide gels (0.75 mm thickness) were used for the detection of mutations on the *PS* genes. 10 ml of 25 per cent polyacrylamide solution was mixed in 8 ml dH_2O and 2 ml 10X buffer. The polymerization was initiated with the addition of 125 µl 10 per cent APS and 12.5 µl TEMED to the mixture. The gel solution was poured between two glass plates, cleaned with water and alcohol and allowed to polymerize for one hour.

8 µl of PCR product was mixed with 8 µl of denaturing dye and the mixture was denatured at 95ºC for 5 minutes. The denatured sample was applied into the wells, and the gels were run in a vertical electrophoresis equipment at 4ºC and in 1X TBE buffer according to the conditions given in Table 3.5.

4.3.1.3. Visualisation of the SSCP Bands by Silver Staining. Polyacrylamide gels were silver stained in a plastic box according to following procedure:

Fixation: in 100 ml of10 peer cent ethanol for 10'

Oxidation: in 100 ml of Nitric acid solution for 3'

Washing: in 100 ml of dH_2O for 1'x 3 times

Staining: in 100 ml of Silver nitrate solution for 40'

Washing: in 100 ml of dH_2O for 1'

Development: in 100 ml of Sodium carbonate solution until the bands were seen.

Stopping: in 100 ml of 10 per cent Acetic acid for 3'

Washing: in 200 ml of dH_2O for 1'

The stained gels were covered with wet cellophane sheets and were allowed to dry at room temperature, using Hoefer Easy Breeze Drying Frame Gel Drier (Pharmacia Biotech, Sweeden).

Table 3.4. PCR conditions used in the analysis of *PS* genes

Gene	Exon	$[Mg^{+2}]$ mM	Annealing temp.	Number of cycles
PS1	1A	1	60	40
	1B	1.5	60	32
	2	1.5	56	32
	3	2	58	32
	4	1.5	58	32
	5	1.5	58	32
	6	2	52	32
	7	1.5	58	32
	8	2	46	32
	9	1.5	58	32
	10	1.5	58	32
	11	1.5	58	32
	12	1.5	52	32
PS2	1[1]	1.5	62	38
	2	2	64	30
	3	1.5	57	30
	4	1.5	54	30
	5	1.5	60	30
	6	1.5	58	30
	7	1.5	64	30
	8	1.5	63	30
	9	1.5	62	30
	10	1.5	58	30
	11	1.75	62	32
	12	1.5	64	30

[1] 10 per cent DMSO was added in the amplification of this exon.

4.3.2. MAPT Gene

4.3.2.1. Amplification of Exons 9, 10, 11, 12, 13 of MAPT Gene by PCR. PCR amplification for the identification of mutations on the *MAPT* gene, was carried out using the following procedure: 1 µl of genomic DNA was amplified in a 25 µl reaction mixture, containing 2.5 µl of 10X PCR reaction buffer, 1.5 µl of $MgCl_2$, 0.2 µl of 25 mM dNTP mix, 1 µl of each primer (from 20 µM stock solution), 0.25 µl of Taq polymerase (5Units/µl). The final volume of the PCR mixture was completed to 25 µl with ultra pure dH_2O.

The PCR procedure, containing two cycling phases, (Touch-down PCR) was used for the amplification of *MAPT* exons. In the first phase, the annealing temperature decreases from 60ºC to 50ºC at every cycle. In the second phase, the annealing temperature is kept constant at 50ºC:

Initial Denaturation at	: 95 ^oC	5’	
Phase I			
Denaturation at	: 95 ^oC	30’’	20 cycles
Annealing at	: 60-50 ^oC	30’’	
Extension at	: 72 ^oC	45’’	
Phase II			
Denaturation at	: 95 ^oC	30’’	17 cycles
Annealing at	: 50 ^oC	30’’	
Extension at	: 72 ^oC	30’’	
Final Extension at	: 72 ^oC	4’	

4.3.2.2. SSCP Analysis by Using Polyacrylamide Gel Electrophoresis. SSCP analysis of the *MAPT* exons was done as mentioned in Section 4.3.1.2.

4.3.2.3. Visualisation of SSCP Bands by Silver Staining. Polyacrylamide gels were silver-stained as described in Section 4.3.1.3.

When a sample with an aberrant pattern was detected on the polyacrylamide gels, the experiment was repeated in order to avoid a false-positive, and then prepared for sequencing.

4.4. PCR Purification and Preparation of Samples for DNA Sequencing

The QIAquick PCR Purification Kit Protocol was applied to purify the PCR products from excess primers, dNTPs, polymerases and salts prior to DNA Sequencing. Five volumes of Buffer PB was added to 40 µl PCR product and mixed. The QIAquick spin column was placed in a two ml collection tube, provided by the kit. The sample was applied to the QIAquick column and spinned for one minute at 13 000 rpm. The flow-through was discarded and the QIAquick column was placed back into the same tube. After the addition of 0.75 ml Buffer PE to the QIAquick column for washing, the sample was spinned for one minute at 13 000 rpm. The flow-through was discarded and the QIAquick column was placed back into the same tube. The column was spinned for an additional one minute at 13 000 rpm. The QIAquick column was placed in a clean 1.5 ml microcentrifuge tube. 30 µl Buffer EB (10mM Tris-Cl, pH 8.5) was added to the center of the QIAquick membrane and the column was spinned for one minute at 13000 rpm in order to elute the DNA. The quality and quantity of the purified PCR products were determined on a two per cent agarose gel.

Table 3.5. Electrophoresis conditions for the SSCP analysis of *PS* and MAPT exons

Region	**Watt/mAmper**	**Duration**
PS1ex1A	15W	2:30
PS1ex1B	12W	2:30
PS1ex2	30mA	2:00
PS1ex3	15W	2:30
PS1ex4	15W	2:30
PS1ex5	15W	2:30
PS1ex6	25mA	1:15
PS1ex7	15W	2:30
PS1ex8	30mA	2:00
PS1ex9	15W	2:30
PS1ex10	20mA	3:00
PS1ex11	15mA	2:00
PS1ex12	15W	2:30
PS2ex1	15W	2:30
PS2ex2	20mA	2:30
PS2ex3	15W	3:30
PS2ex4	14W	2:30
PS2ex5	15W	2:30
PS2ex6	35mA	2:45
PS2ex7	15W	2:30
PS2ex8	15W	2:30
PS2ex9	13W	2:30
PS2ex10	15W	2:30
PS2ex11	15W	2:30
PS2ex12	15W	2:30
MAPTex9	13W	3:00
MAPTex10	13W	2:30
MAPTex11	13W	3:00
MAPTex12	13W	2:30
MAPTex13	13W	3:00

5. RESULTS

5.1. Alzheimer's Disease

In the framework of this thesis, the entire coding regions of the *PS1* and *PS2* genes were screened in six unrelated patients from five families with a history of early onset AD, who presented with concerns of dementia.

Cases were considered familial, when at least one first degree relative suffered from dementia. Among familial AD cases, the pedigree was considered to segregate with autosomal dominant probable AD, if at least three patients with dementia were reported in three generations.

A four-step approach was employed in the analysis of these patients:

1. Initially, the entire coding regions, 13 exons of the *PS1* gene and 12 exons of the *PS2* genes, were amplified.

2. Following the PCR amplifications, SSCP analyses were performed in order to detect the possible mutations on these genes. PCR products of *PS1* and *PS2* exons of individuals, who were previously shown not to carry a mutation, were used as negative controls and run on SSCP gels next to the samples to be analyzed.

3. When an aberrant pattern was seen on the gel, the SSCP analysis was repeated with a new PCR product, in order to avoid false-positive results.

4. Finally, if the SSCP gels repeatedly showed the same aberrant pattern, samples were prepared for sequencing.

5.1.1. Analysis of AD Family 1

The index case of Family 1 was a 38-years-old male, when he was referred to our laboratory from Gülhane Military Medical Academy (GATA). According to his medical history, he was showing deficits in intellectual functions, attention, and concentration abilities. His computerized tomography, standard electroencephalography (EEG), and brain magnetic resonance imaging (MRI) results were normal. Brain mapping revealed significant reduction in memory at the right hemisphere of his brain, which was shown to be improved six months later.

The parents of this patient were still healthy and had no signs of AD. However, his father's brother and his mother's sister died because of late onset AD. He had also another relative who was institutionalized with AD.

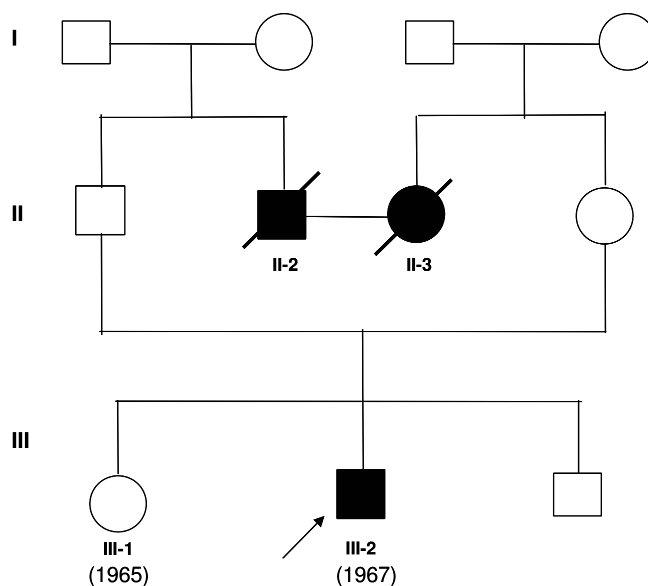

Figure 5.1. Pedigree: AD Family 1

Other family members, with AD diagnosis had deceased, thus only the index case was available for analysis (Figure 5.1). All the exons of *PS1* were investigated, but no mutation could be detected. An aberrant pattern in exon 5 of the *PS1* gene turned out to be a false-positive (Figure 5.2.A).

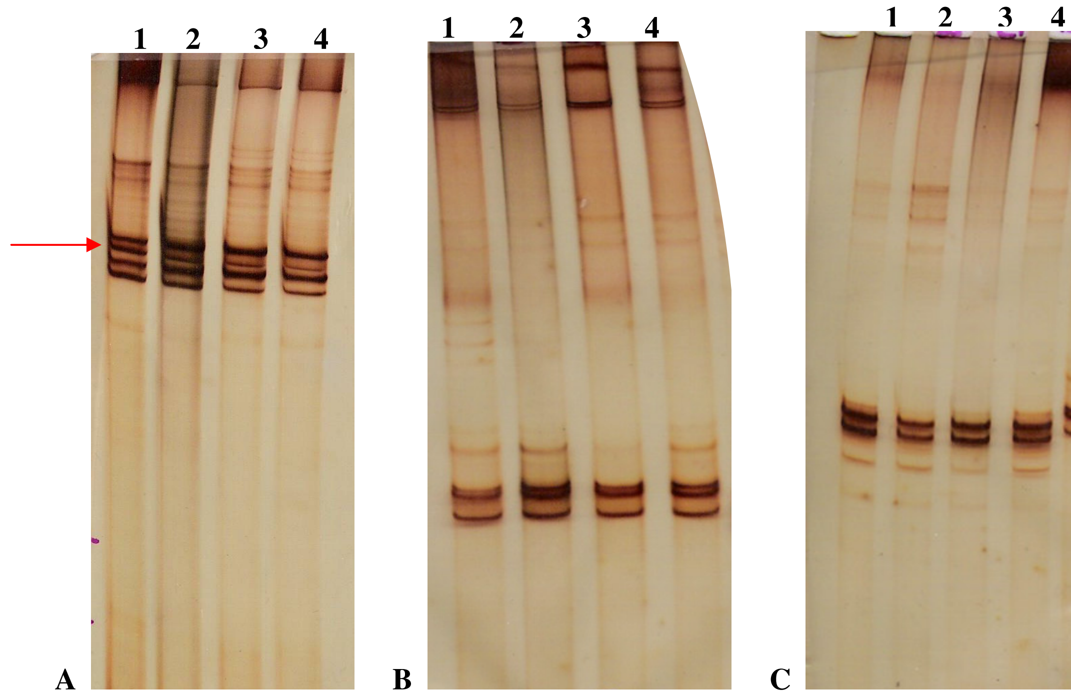

Figure 5.2. SSCP analysis of proband in AD pedigree 1

A) SSCP analysis of the *PS1* exon 5 (1 shows the aberrant pattern of *PS1*/exon 5 of the patient, where a thicker second band is observed. 2, 3, and 4 are the results of control samples) B) SSCP analysis of *PS1*/exon 8 C) SSCP analysis of *PS1*/exon 9 (Lane 1: Proband, Family 1; lanes 2-4: control samples)

5.1.2. Analysis of AD Family 2

The index case of family 2 was referred to our laboratory from the Neurology Department of Akdeniz University Medical School. He had complaints of memory loss which were prominent in neuropsychological tests, since he was 36 years old. Behavioral problems, such as apathy and irritability were accompanied by decline in daily living activities.

The patient also had a brother who showed similar clinical features but died early in an accident. The patient was subjected to SSCP analysis for entire coding regions of *PS1* and *PS2* (Figure 5.3). Since no aberrant pattern was observed on the polyacrylamide gels by screening the entire coding regions of *PS1* and *PS2*, the analysis was discontinued (Figures 5.6.-9.).

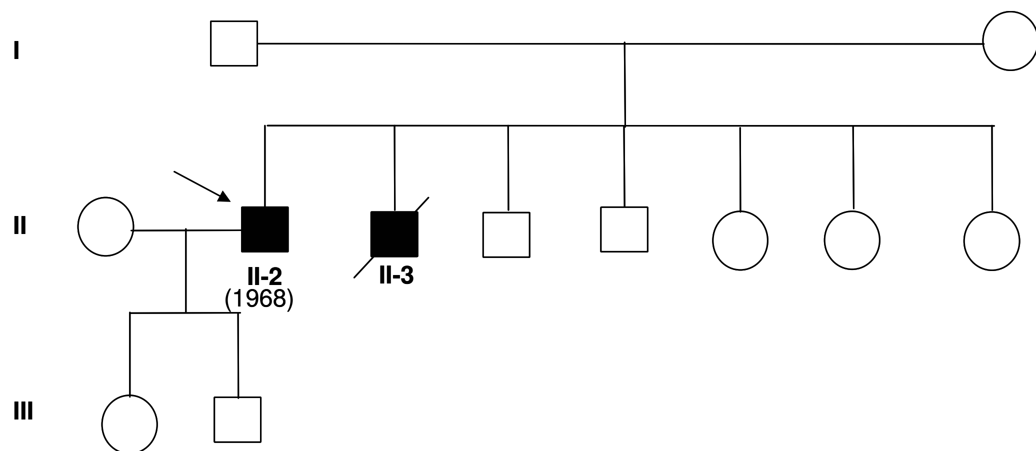

Figure 5.3. Pedigree: AD Family 2

5.1.3. Analysis of AD Family 3

Two affected sibs in Family 3 were referred to our laboratory from Neurology Department of Celal Bayar University Medical School (Figure 5.4). Both of them had early onset dementia. There was no systemic disease that might underlie the dementia. No other affected family members were present.

The family did not fulfill the NINCDS/ADRDA criteria to full extent; also no vertical transmission was present. However, since the affected members were sibs, both with a history of dementia, they were analyzed by SSCP for all exons of the *PS1* and *PS2* genes, however, no aberrant pattern was seen (Figures 5.6.-9.).

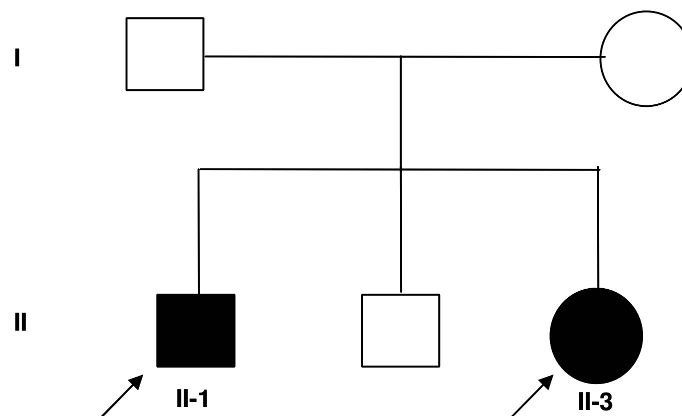

Figure 5.4. Pedigree: AD Family 3

5.1.4. Analysis of AD Family 4

Proband of Family 4, originating from Kars, was referred to our laboratory from the Neurology Department of Marmara University Medical School.

The 36-years-old patient showed marked deficits in memory and learning for six years. The course of the disease was rapid and devastating; his MMSE (Mini Mental Status Examination) score was 5/30, indicating severe cognitive impairment.

PS1 and *PS2* exons of the patient were amplified and screened by SSCP. Since there was no abnormal pattern in the SSCP analysis, the sample was not investigated further (Figures 5.6.-9.).

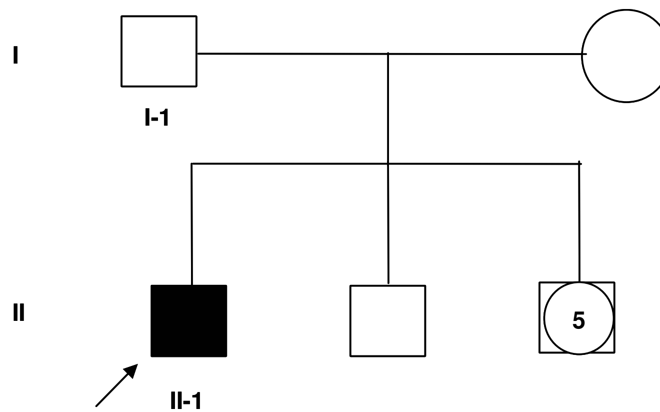

Figure 5.5. Pedigree: AD Family 4

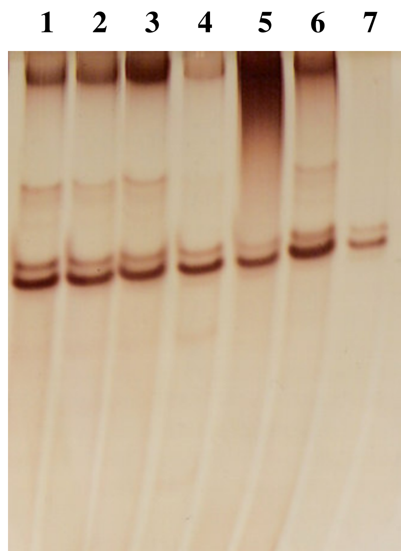

Figure 5.6. SSCP analysis of *PS1* exon9

(Lanes 1-4: Probands, Families 2, 3, 3, 4; lanes 5-7: control samples)

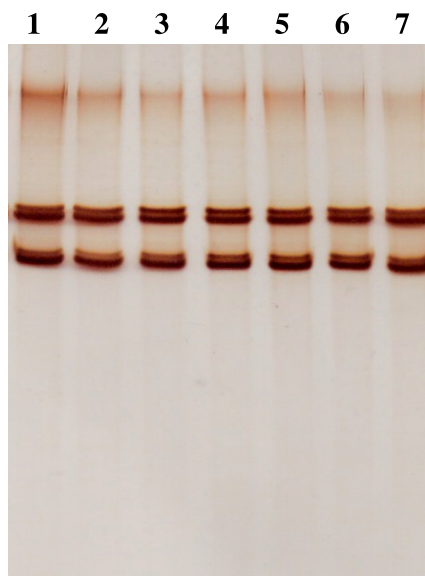

Figure 5.7. SSCP analysis of *PS2* exon6

(Lanes 1-4: Probands, Families 2, 3, 3, 4; lanes 5-7: control samples)

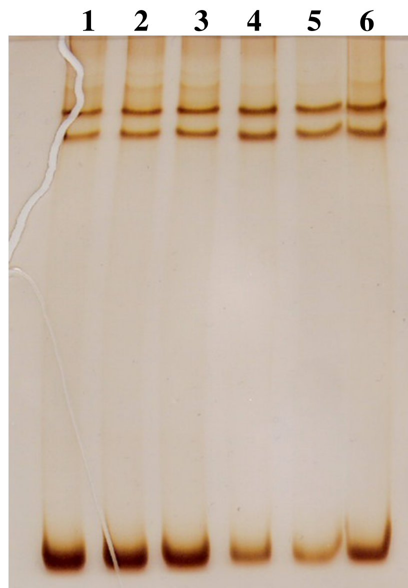

Figure 5.8. SSCP analysis of *PS1* exon1B

(Lanes 1-4: Probands, Families 2, 3, 3, 4; lanes 5, 6: control samples)

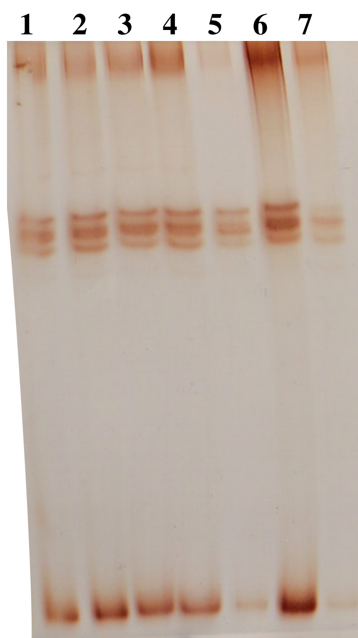

Figure 5.9. SSCP analysis of *PS1* exon11

(Lanes 1-4: Probands, Families 2, 3, 3, 4; lanes 5-7: control samples)

5.1.5. Analysis of Family 5

The proband of Family 5 (III-1), a 49-years-old male, was referred to us from the Neurology Department of İstanbul Medical School with mild memory deficits. Since four years, he showed a progressive decline in cognitive functions and in daily living activities. Neuropsychological evaluation revealed an overall mental decline (MMSE score 22/30). MRI scan of the brain displayed moderate atrophy of the temporal lobes. His disease story revealed that his mother (II-2) and maternal aunt (II-3), who died at their fifties, were described to have developed symptoms of dementia in their late forties. His maternal grandmother (I-2) had also dementia and died at 50 years of age. The family fulfilled the NINCDS/ADRDA criteria satisfactorily, and the inheritance pattern was consistent with autosomal dominant transmission for three generations.

The only affected member (III-1) was screened for the *PS1* and *PS2* exons (Figure 5.10).

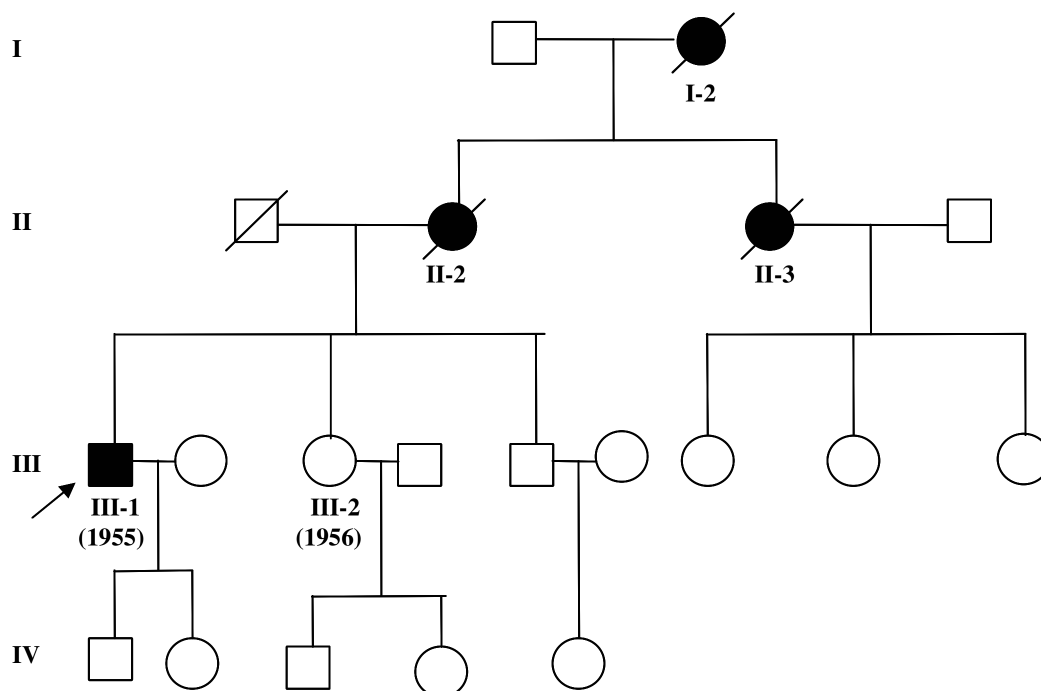

Figure 5.10. Pedigree: AD Family 5

The analysis of the *PS1* gene of the index case in Pedigree 5 revealed an abnormal migration pattern in exon11 (Figure 5.11).

Figure 5.11. Abnormal migration of the sample on SSCP gel (arrow)

Direct sequencing of exon11 of the *PS1* gene from the patient revealed a novel GCC-ACC transition corresponding to an Ala396Thr mutation in the HL-VII domain of the *PS1* protein (Figure 5.12).

The 48-years-old sister of the patient, who did not show any clinical signs of AD was also subjected to DNA analysis upon her own request. DNA analysis was performed after genetic counseling and informed consent was obtained; the result revealed that she did not carry the above mutation.

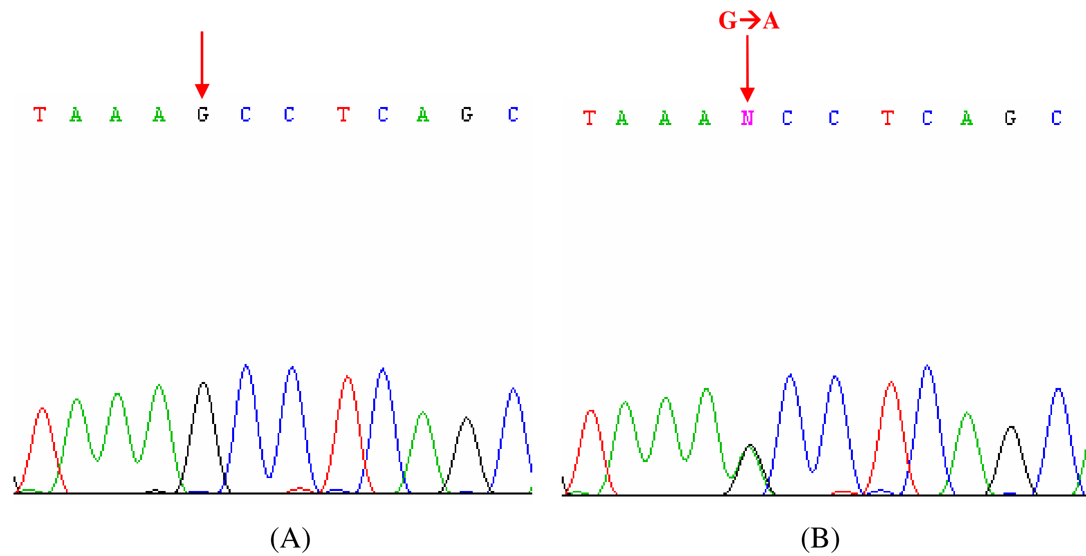

Figure 5.12. Sequencing of the *PS1*/Exon11 with the forward primer A) Wild-type sequence of the control sample B) Sequence of the index case showing the G→A substitution

To confirm this result, a new PCR was performed and sequencing with the reverse primer showed the novel mutation in the proband (Figure 5.13).

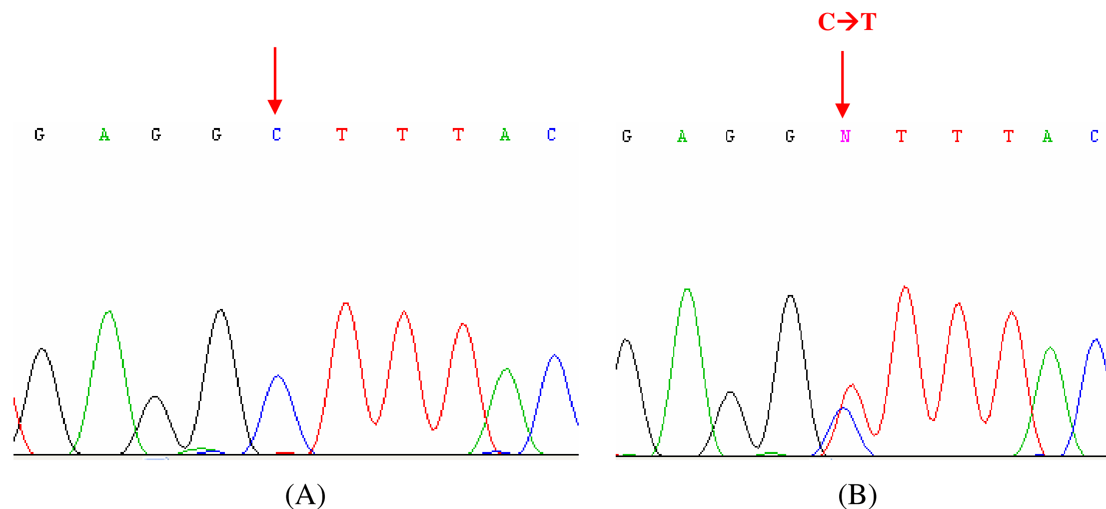

Figure 5.13. Sequencing of the *PS1*/Exon11 with the reverse primer A) Wild-type sequence of the control sample B) Sequence of the index case showing the C→T transition

5.2. Frontotemporal Dementia

In the framework of this study, exons 9, 10, 11, 12, and 13, being the mutational hotspot regions of the *MAPT* gene, were analyzed in four affected individuals from three unrelated families with a history of FTD. Patients with first-degree relatives with dementia of frontotemporal type were considered as familial FTD.

A two-step approach was employed in the analysis of these patients:

1. Initially, exons 9-13 of the *MAPT* gene were amplified.

2. Following the PCR amplifications, SSCP analysis was performed in order to detect the possible mutations on this gene. PCR products of *MAPT* exons of healthy individuals were used as negative controls and run on SSCP gels next to the samples to be analyzed.

5.2.1. Analysis of FTD Family 1

The proband of the FTD Family 1 was referred to our laboratory from the Neurology Department of Marmara University Medical School. The patient had complaints of weakness of both upper and lower limbs, restricted memory and aphasia which started three years ago.

The ten years older brother of the patient had shown behavioral alterations and deficits in memory, which progressed within two years and finally resulted in death due to AD. His other siblings did not show the symptoms of the disease, however cardiovascular disease was common in this family.

The proband was subjected to *MAPT* gene analysis by SSCP, but no mutation was found in the *MAPT* gene.

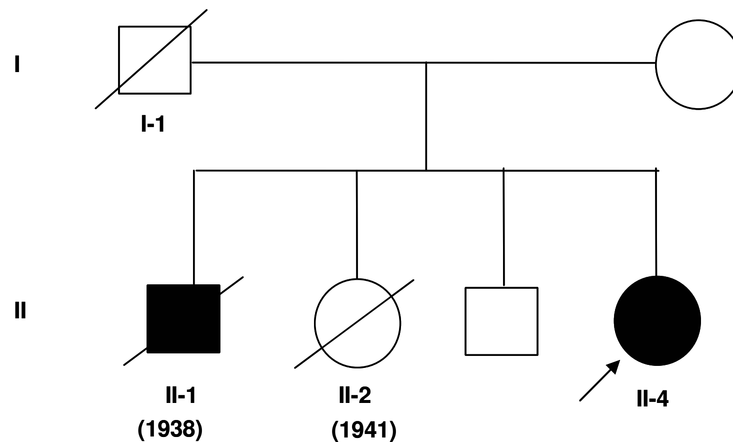

Figure 5.14. Pedigree: FTD Family 1

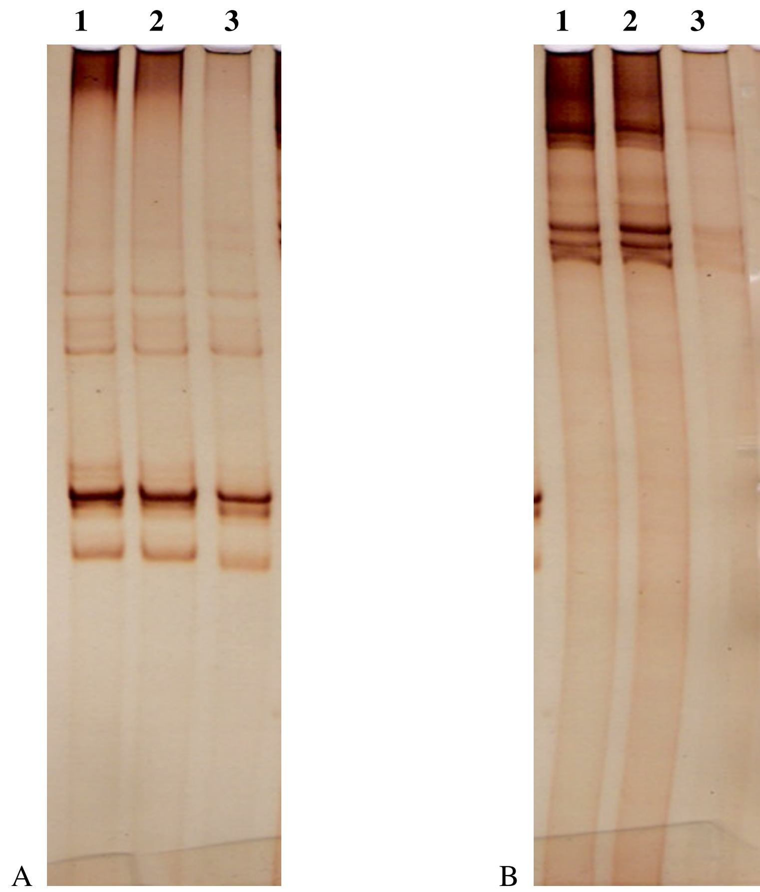

Figure 5.15 A) SSCP analysis of exon 11 of the *MAPT* gene B) SSCP analysis of the exon 13 of the *MAPT* gene (Lane 1: Proband, Family 1; lanes 2, 3: control samples)

5.2.2. Analysis of FTD Family 2

The proband of FTD Family 2, with a positive family history of FTD, was referred to our laboratory from the Neurology Department of Istanbul Medical School.

The father and brother of the proband had progressive dementia, which started at the age of 50. Cranial MRI examination of the patient revealed atrophy of the frontal lobe. Since the other affected individuals of the family deceased earlier, only the index case was available for analysis. SSCP analysis of the exons, analyzed in the framework of this study, did not show an abnormal migration pattern on SSCP gels.

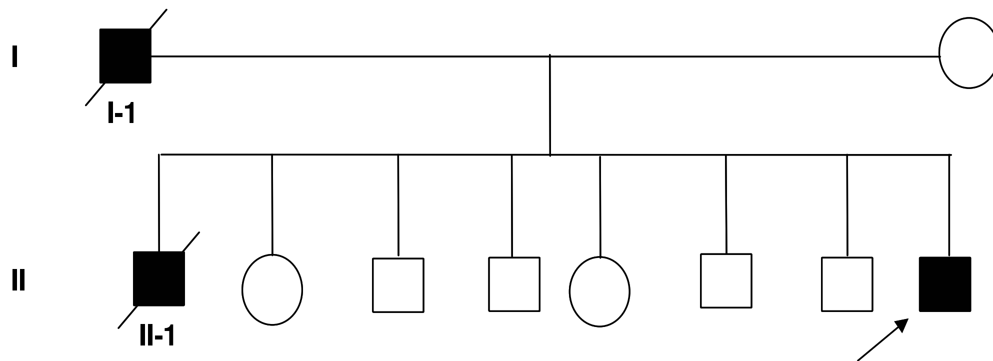

Figure 5.16. Pedigree: FTD Family 2

5.2.3. Analysis of FTD Family 3

There are two probands in FTD Family 3, who were referred to our laboratory from the Neurology Department of Marmara University Medical School with a clinical diagnosis of FTD.

The *MAPT* exons 9-13 of both probands were amplified and run on polyacrylamide gels. However, no aberrant pattern was seen in any exon, and the analyses were terminated.

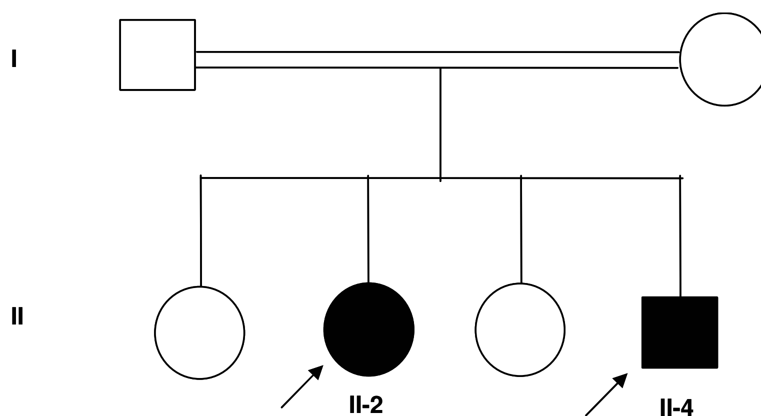

Figure 5.17. Pedigree: FTD Family 3

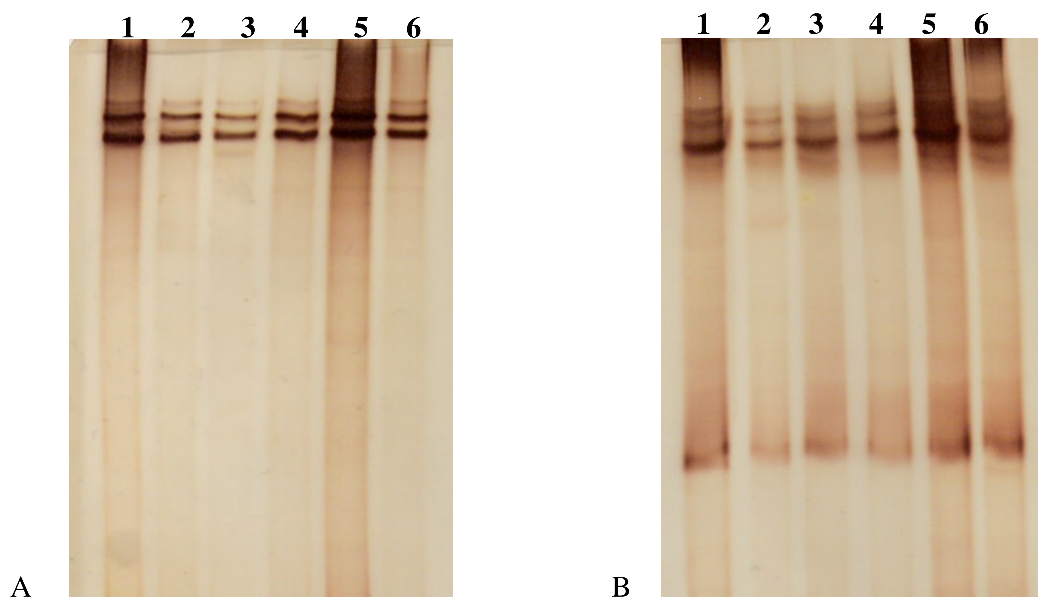

Figure 5.18. A) SSCP analysis of exon12 of the *MAPT* gene B) SSCP analysis of exon13 of the *MAPT* gene (Lanes 1-3: Probands, Families 2, 3, 3; lanes 4-6: control samples)

The results of six AD and four FTD families are compiled in Table 5.1. and Table 5.2.

Table 5.1. EOFAD samples and their SSCP and sequencing results

Sample	Probable Clinical Diagnosis	PS1 SSCP	PS2 SSCP	PS1 Seq.	PS2 Seq.
AD Fam. 1/ III-2	EOAD	**Abrr**	**X**	**N**	**-**
AD Fam. 2/ II-2	EOAD	**X**	**X**	**-**	**-**
AD Fam. 3/ II-1	EOFAD	**X**	**X**	**-**	**-**
AD Fam. 3/ II-3	EOFAD	**X**	**X**	**-**	**-**
AD Fam. 4/ II-1	EOAD	**X**	**X**	**-**	**-**
AD Fam. 5/ III-1	adEOFAD	**Abrr**	**X**	**Ala396Thr**	**-**

Table 5.2. FTD samples and their SSCP and sequencing results

Sample	Probable Clinical Diagnosis	MAPT SSCP	MAPT Sequencing
FTD Fam.1/ II-4	FTD	**X**	**-**
FTD Fam.2/ II-8	Familial FTD	**X**	**-**
FTD Fam.3/ II-2	Familial FTD	**X**	**-**
FTD Fam.3/ II-4	Familial FTD	**X**	**-**

EOAD: Early onset AD

EOFAD: Early onset familial AD

adEOFAD: Autosomal dominant early onset AD

Abrr: Aberrant SSCP pattern

X: Normal SSCP pattern

N: Normal sequencing pattern

- : Not sequenced, because no aberrant pattern was observed in SSCP gel

6. DISCUSSION

AD is the most common form of age-related dementia, characterized by progressive decline in cognitive functions, such as thinking and formation of memory. Besides, AD is accompanied by having unusual behavior and changes in personality.

Prevalance of AD increases with age. It has been estimated that in 50 years, approximately 30 per cent of the population in well-developing countries will be aged 65 years or older. According to this, it is thought that worldwide 18 million people will develop AD. The early onset form of AD which represents only a small fraction of all AD cases (≤5 per cent) shows an onset age below 60 years. EOFAD is transmitted within the families in an autosomal dominant fashion.

FTD is the most common neurodegenerative dementia syndrome after AD, with a mean onset age of 52 years. The specific cause of FTD is still unknown. The clinical features of FTD are, increasing cognitive problems with language deficits, alterations in personality and behaviour. In the course of the disease, patients finally become aphasic.

Since there is no specific laboratory test either for AD or for FTD, the differential diagnosis of these two syndromes is very difficult. Some neuroimaging methods such as MRI, computerized tomography (CT), single photon emission computed tomography (SPECT), and positron emission tomography (PET) may help clinicians to distinguish FTD from AD. Among these imaging techniques, MRI is thought to be more sensitive than CT in evaluating patients with brain pathology. However, both AD and FTD patients may have normal results on these scans. Thus, molecular analysis in familial cases turns out to be very important.

Although the pathophysiology of AD has not been understood yet clearly, it is known that the *PS1* and *PS2* genes play an important role in predisposing to disease onset and in modifying the progression of the disease. The *PS1* and *PS2* genes are closely related to each other and encode proteins called with the name of the genes; they are located on intracellular membranes, such as the nuclear envelop, the endoplasmic reticulum and the

Golgi apparatus. The 67 per cent homologous proteins PS1 and PS2 contain multiple transmembrane domains.

158 mutations on the *PS1* gene and 11 mutations on the *PS2* gene were reported up to date; most of these are missense mutations, although there are also some small deletions and insertions. Because no correlation between the site of the mutation on the *PS1* gene and its phenotype is reported, it is suggested that the site of the *PS1* mutations are largely unimportant. Most mutations act as toxic gain of function mutations affecting the γ-secretase activity. Mutations on the *PS1* and *PS2* genes are sufficient to cause AD by altering the cleavage of APP and increasing the ratio of Aβ42 to Aβ40.

The *MAPT* gene, mutations on which are responsible for FTD, encodes Tau, a microtubule binding protein, that is believed to be important for the assembly and stabilization of microtubules. Tau is abundant in nerve cells, where it is expressed particularly in axons; in the tauopathies, Tau is redistributed to the cell body and dendrites.

Fifty eight mutations were identified on the *MAPT* gene up to date, and these are either missense or deletion mutations or silent mutations in the coding regions, and intronic mutations. The identification of tau mutations has provided clear evidence, that Tau dysfunction can result in neurodegeneration, by either disrupting Tau binding to microtubules, or altering the tendency of Tau to form aggregates.

Six EOAD and four FTD patients were investigated in the framework of this study for their genotypes. For this purpose, the entire coding regions of the *PS1* and *PS2* genes were investigated in the AD patients. Exons 9, 10, 11, 12 and 13 of the *MAPT* gene were analyzed in the FTD patients.

With the methodology applied, SSCP and genomic sequencing, one AD patient's disease was shown to be attributable to a *PS1* mutation, which had not been described before in the literature.

6.1. A Novel PS1 Mutation

The patient with a clear EOFAD history, segregating since at least three generations in the family, had an abnormal migration pattern in the exon11 of the *PS1* gene. This abnormal migration pattern was reproduced several times in new amplification reactions and SSCP gels. Upon confirmation, the sample was prepared for sequencing, which revealed the transition of a GCC to an ACC in exon11 (Figure 6.1.).

ggttgagtagggcagtgatatttttgaattgtgaaatcatancaaagagtgaccaactttttaata
tttgtaacctttcctttttag**GGGGAGTAAAACTTGGATTGGGAGATTTC**
ATTTTCTACAGTGTTCTGGTTGGTAAAGCCTCAGCAACAGC
CAGTGGAGACTGGAACACAACCATAGCCTGTTTCGTAGCCA
TATTAATTgtmmstatacactaataagaatgtgtcagagctcttaatgtcmaaactttga
ttacacagtccctttaa

Figure 6.1. The sequence of the *PS1* exon11. The highlighted letters represent the forward and reverse primers, the capital letters represent the exonic sequences. Green highlighted G shows the location of the identified point mutation

At protein level, this change results in the substitution of the nonpolar alanine with the polar threonine at position 396 of PS1. The mutation is located at the HL-VII domain of the PS1 protein (Figure 6.2).

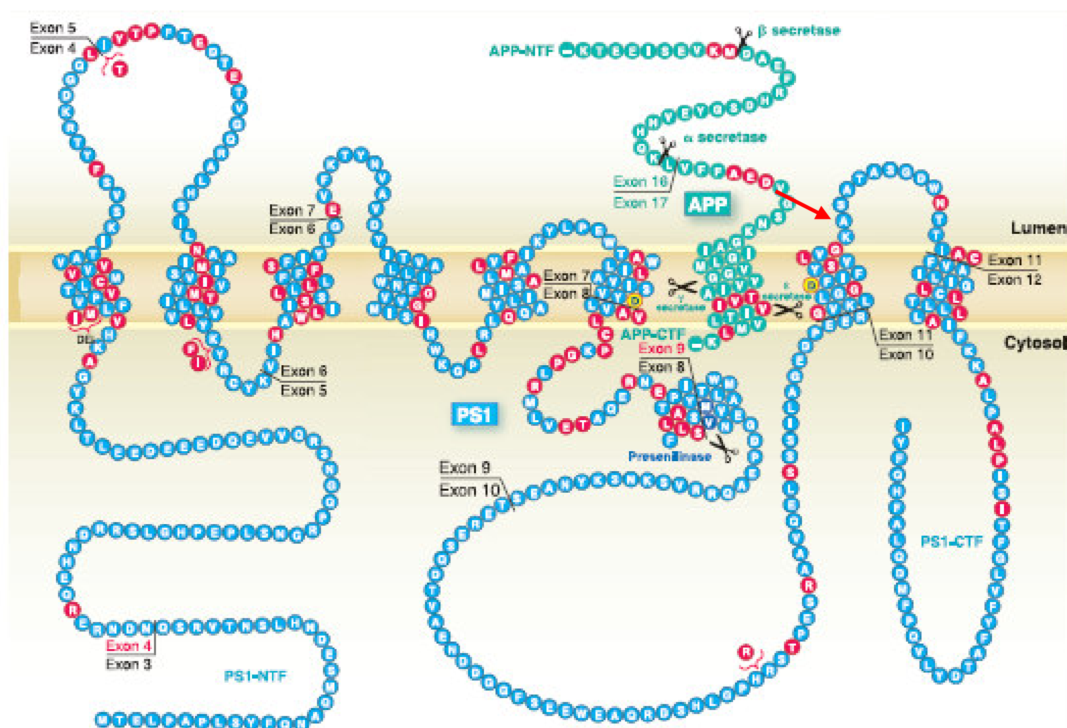

Figure 6.2. PS1 protein: The novel mutation (Ala396Thr), located in the cytosolic part, is depicted by a red arrow (Hardy and Selkoe, 2002)

This Ala396Thr substitution has not been reported thus far in the literature. It is also not present in the NCBI database. Since it was not shown in 26 Turkish control subjects in a study performed previously in our laboratory (Yokeş B., 1999), it is assumed that it is a novel mutation, and not a polymorphism. A further proof was obtained by the favorable result of the unaffected sister of the patient, who seemed to have escaped the disease on neurological examination. She was subjected to DNA analysis upon own request. For this purpose genetic counseling was offered and informed consent was obtained. She was found not to carry the mutation.

The substitution of the non-polar alanine by the polar threonine in the coding region of PS1 is thought to cause a change in the structure of the PS1 protein resulting in an alteration in APP cleavage by constraining the structure of the protein.

6.2. Patients with Unexplained Genotypes

In the remaining five EOAD and four FTD patients, the SSCP patterns were found to be insignificant, thus their analyses were interrupted at this level. The failure of molecular diagnosis in these patients can have several reasons:

- Numerous early onset families do not show mutations in any of the three known genes (*APP, PS1, PS2*), despite extensive sequencing attempts of open reading frames (ORFs) and intronic regions.

- There are several additional key proteins beyond APP, PS1, PS2 and Tau, involved in EOAD and FTD.

- The mutation screenings of *PS1, PS2* and *MAPT* were performed by SSCP analyses. The sensitivity of SSCP is not 100 per cent. Although the conditions were optimized well, and the gels were run several times and evaluated carefully, there can always be unfavorable sequence constellations in the *PS1, PS2* and *MAPT* exons that can be missed by SSCP analysis. The development of more powerful and efficient genotyping and analytic tools will enable us to solve the genetics of complex diseases, when more reliable genetic risk profiling will become available.

- The clinical characteristics of EOAD and FTD may overlap with several other neurodegenerative diseases, such as cerebral amyloid angiopathy, progressive supranuclear palsy, corticobasal degeneration, spastic paraparesis and Pick's disease. Thus, a reliable clinical and neuropathological diagnosis and stringent criteria, ascertaining the EOAD and FTD diagnoses are crucial.

More than a decade after the discovery of the *PS1* and *PS2* genes, the requirement for molecular testing in affected families is becoming increasingly common. The relative contributions of *PS1* and *PS2* gene mutations to EOAD development are not yet well-defined; but they are important to ensure efficient assistance and genetic counseling in affected families. Although in a previous report, the efficiency of *PS1* and *APP* mutation

analyses in 34 EOAD families was claimed to be >50 per cent (Raux et al., 2006), the estimates in other studies, however on smaller sample sizes, were much lower.

7. CONCLUSIONS

In the framework of this thesis, in the six EOAD families under investigation, only one was shown to be the result of a pathogenic *PS1* mutation, previously not reported in the literature. Retrospectively, we claim, that this was the only family in our cohort, who fulfilled the full criteria for probable or definite EOAD. Keeping this fact in mind, and taking into account the tedious analyses of the *PS1* and *PS2* genes, we can conclude that the clinical and neuropathological examination of AD families must confirm a probable EOAD diagnosis in at least three generations with fulfillment of the international criteria; otherwise, negative results will be difficult to avoid in molecular analysis.

A better understanding of the complex etiology that underlies EOAD and FTD may be achieved through a multidisciplinary approach that combines clinical and neurophysiological characterization of diseases with molecular investigations of genetic components. Newly developing, high resolution capacity imaging methods, that use specific antibodies for the detection of apoptosis in brain, contribute to the early and definite diagnosis of neurodegenerative disorders. Additionally, linkage analysis with large families will determine novel candidate genes and SNP genotyping of patients by microarray methodology will permit to examine hundreds of polymorphisms in candidate genes, in the same time allowing to obtain more data.

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