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**A MOLECULAR GENETIC STUDY OF FACTORS INVOLVED IN ALZHEIMER'S
DISEASE**

Doctoral dissertation

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ABSTRACT

Alzheimer's disease (AD) is the most common cause of progressive neurological disorder leading to dementia. It is neuropathologically characterized by extracellular and perivascular deposits of amyloid beta peptide and by the generation of intracellular neurofibrillary tangles. AD is subdivided into early and late onset forms and it has a genetic aetiology, which is most evident in the case of familial early onset AD (onset age before 65 years). At present, mutations in amyloid precursor protein (APP), presenilin 1 (PSEN-1), and presenilin 2 (PSEN-2) genes are known to cause the autosomal dominant form of early onset AD while the apolipoprotein E (ApoE) ϵ 4 allele has been associated with an increased risk of developing both early and late onset AD.

The purpose of this study was to assess the genetic components involved in the early and late onset AD in Finland. Despite the well-established effects of the APP, PSEN-1, PSEN2 and ApoE genes in early and late onset AD, it is obvious that additional susceptibility genes are involved. In order to detect these novel chromosomal loci, a genome-wide linkage disequilibrium mapping was performed using late onset AD patients and age-matched control subjects from a geographically restricted area in Eastern Finland. In addition, the effects of different susceptibility and causative genes such as butyrylcholinesterase (BChE) K variant, ApoE promoter -491A/T polymorphism, and PSEN-1 were examined among Finnish early and late onset AD patients.

The major findings of this study were as follows: 1) Identification of a novel 4.6-kb genomic deletion in PSEN-1 gene, which leads to the exclusion of exon 9 in an early onset AD family. This germline alteration was clearly a causative mutation for AD and the clinical and neuropathological phenotypes of patients were those of typical AD without indications of spastic paraparesis or 'cotton wool' plaques. The underlying recombination mechanism in this genomic deletion was considered to involve an *Alu* core sequence-stimulated non-homologous rearrangement. 2) Although the E318G substitution in the PSEN-1 gene was found to be a non-causative mutation in AD, the frequency of this substitution was increased both in the sporadic and familial AD patient groups suggesting that E318G could be a risk factor for AD. 3) BChE K variant and ApoE ϵ 4 alleles do not act in synergy in Finnish late onset AD patients. Instead, reduced BChE K allele frequency among AD patient group under 75 years of age and carrying the ApoE ϵ 4 allele points to a protective effect of the BChE K variant allele in this subgroup. 4) ApoE promoter -491A/T polymorphism did not reveal significant differences between late onset AD patients and age-matched controls. Consistent with previous studies, -491 A and ApoE ϵ 4 alleles were found to be in linkage disequilibrium indicating that the ApoE ϵ 4 status is still the strongest predictor of risk in Finnish late onset AD patients. 5) Initial genome-wide linkage disequilibrium mapping with 366 polymorphic microsatellite markers revealed 22 chromosomal regions which were associated with AD with P-values < 0.05. Comparison of single allele frequencies of the microsatellite markers in AD and control groups revealed the presence of both possible risk and protective alleles. Screening of the 22 LD regions with additional microsatellite markers revealed that eight chromosomal loci in 1p36.12, 2p22.2, 3q28, 4p13, 10p13, 13q12, 18q12.1 and 19p13.3 were associated with AD in more than one microsatellite marker. These chromosome regions found to be associated with AD in the present study will provide the primary targets for future genetic and functional studies into AD.

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Keep the Faith

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ABBREVIATIONS

A2M	Alpha-2-macroglobulin
A β	Amyloid beta peptide
AChE	Acetylcholinesterase
ACh	Acetylcholine
AD	Alzheimer's disease
AM	Activated microglia
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
BChE	Butyrylcholinesterase
CAA	Cerebral amyloid angiopathy
cM	centiMorgan
$\Delta 9$	Deletion of exon nine
ER	Endoplasmic reticulum
IBD	Identical-by-descent
LD	Linkage disequilibrium
MLS	Multipoint maximum lod score
MPO	Myeloperoxidase
NFT	Neurofibrillary tangle
NT	Neuropil thread
PCR	Polymerase chain reaction
PHF	Paired helical filament
PSEN	Presenilin
QTL	Quantitative trait loci
RA	Reactive astrocyte
RFLP	Restriction fragment length polymorphism
RT-PCR	Reverse transcriptase polymerase chain reaction
SNP	Single-nucleotide polymorphism
TGN	Trans Golgi network
TM	Transmembrane domain

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications that are referred to in the text by the Roman numerals **I-VI**.

- I.** Hiltunen M, Helisalmi S, Mannermaa A, Alafuzoff I, Koivisto AM, Lehtovirta M, Pirskanen M, Sulkava R, Verkkoniemi A, Soininen H: Identification of a novel 4.6-kb genomic deletion in presenilin-1 gene which results in exclusion of exon 9 in a Finnish early onset Alzheimer's disease family: an Alu core sequence-stimulated recombination? *Eur J Hum Genet* 8:259-266, 2000
- II.** Helisalmi S, Hiltunen M, Mannermaa A, Koivisto AM, Lehtovirta M, Alafuzoff I, Ryyänänen M, Soininen H: Is the presenilin-1 E318G missense mutation a risk factor for Alzheimer's disease? *Neurosci Lett* 278:65-68, 2000
- III.** Hiltunen M, Mannermaa A, Helisalmi S, Koivisto A, Lehtovirta M, Ryyänänen M, Riekkinen P Sr, Soininen H: Butyrylcholinesterase K variant and apolipoprotein E4 genes do not act in synergy in Finnish late-onset Alzheimer's disease patients. *Neurosci Lett* 250:69-71, 1998
- IV.** Helisalmi S, Hiltunen M, Valonen P, Mannermaa A, Koivisto AM, Lehtovirta M, Ryyänänen M, Soininen H: Promoter polymorphism (-491A/T) in the APOE gene of Finnish Alzheimer's disease patients and control individuals. *J Neurol* 246:821-824, 1999
- V.** Hiltunen M, Mannermaa A, Koivisto AM, Lehtovirta M, Helisalmi S, Ryyänänen M, Riekkinen P Sr, Soininen H: Linkage disequilibrium in the 13q12 region in Finnish late onset Alzheimer's disease patients. *Eur J Hum Genet* 7:652-658, 1999
- VI.** Hiltunen M, Mannermaa A, Thompson D, Easton D, Pirskanen M, Helisalmi S, Koivisto AM, Lehtovirta M, Ryyänänen M, Soininen H: Genome-wide linkage disequilibrium mapping of late onset Alzheimer's disease in Finland. *Neurology* 57:1663-1668, 2001

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1. INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative condition that is clinically associated with a global cognitive decline such as progressive loss of memory and reasoning whereas at autopsy, one sees a large number of neuritic plaques and neurofibrillary tangles in the cortex of the brain. The exact molecular mechanisms leading to AD are not fully understood, but it has a genetic aetiology, which is the most evident in the case of familial early onset AD. Mutations in the amyloid precursor protein (APP), presenilin-1 (PSEN-1), and presenilin-2 (PSEN-2) genes are responsible for rare autosomal dominant form of the disease, which usually appears before the age of 65 years (Goate et al., 1991, Sherrington et al., 1995, Levy-Lahad et al., 1995, Rogaev et al., 1995). The common pathogenic feature in all of these mutations is the increased production amyloid beta peptide ($A\beta$), which is released from the APP after the proteolytic cleavage of enzymes called β - and γ -secretases. In its 42 amino acid form, $A\beta$ -peptide is the main constituent of neuritic plaques that invade cortical and subcortical areas of AD brains whereas the 40 amino acid form of $A\beta$ -peptide is more prominent in vessel walls of the neuropil and leptomeninges as cerebral amyloid angiopathy (CAA) (Masters et al., 1985, Kang et al., 1987).

Apart from the small proportion of autosomal dominant familial AD patients, the majority of AD patients are sporadic with onset age usually over 65 of years. Sporadic AD patients share similar clinical and neuropathological features as the autosomal dominant AD patients, but the underlying genetic mechanism appears to be more complex. To date, $\epsilon 4$ allele of apolipoprotein E (ApoE) is the only commonly accepted susceptibility factor detected in most populations that increases the risk of early- and late onset sporadic as well as familial AD (Corder et al., 1993, Saunders et al., 1993, van Duijn et al., 1994, Chartier-Harlin et al., 1994). These above-mentioned genes cannot, however, account for the observed familial aggregation of the disease, which is more common in first-degree relatives of AD patients than in the general population, suggesting that additional susceptibility genes exist.

There has been much debate about which are the best procedures for finding novel susceptibility genes for late onset Alzheimer's disease with its acknowledged complex genetic nature. Conventional mapping approaches such as parametric linkage mapping have been proven not to be as efficient as with monogenic Mendelian traits and therefore association-based (Risch and Merikangas, 1996) and multipoint oligogenic analyses (Daw et al., 1999) have been considered as alternative tools. Consistent with previous suggestions, mapping for novel susceptibility genes by virtue of linkage disequilibrium may represent a reasonable choice in genetically isolated populations, because it is more likely to find fewer disease-associated ancestral haplotypes in those populations than in the more admixed, cosmopolitan populations. Also, improved genotyping technology and the increased availability of microsatellite markers and single-nucleotide polymorphisms (SNPs) have made it possible to use association-based strategies in a more efficient way. Thus, combined use of these population and methodological advantages might eventually enable the mapping and cloning of complex disease-associated loci.

The purpose of the present study was to assess the effects of different susceptibility and causative genes among the Finnish early and late onset AD patients. Furthermore, late onset AD patients and age-matched control subjects from a geographically restricted area in Eastern Finland were used in a genome-wide linkage disequilibrium mapping study where our aim was to find novel susceptibility loci for late onset AD.

2. REVIEW OF THE LITERATURE

2.1. Alzheimer's disease (AD)

AD is the most common form of progressive dementia, which affects 5-10% of the population over 65 years of age with the prevalence estimates of AD increasing exponentially with age (Breteler et al., 1992). In addition to age, a positive family history of dementia is considered to be a definite risk factor for AD with a history of depression and severe head trauma being among the possible risk factors (van Duijn et al., 1991a, Jorm et al., 1991, Mortimer et al., 1991).

AD is categorized according to the age of onset into early and late onset forms by using 65 years of age as the cut-off point (Terry and Katzman, 1983). It is clinically characterized by global cognitive decline, including impairment of memory, orientation and intellectual functioning, eventually leading to death. The expected survival time after the disease onset is estimated to be 5 to 12 years and the patients usually die of infectious or other incidental illnesses. Due to the fact that no reliable diagnostic marker in clinical work has been found, the diagnosis of AD is based on the typical course of dementia and exclusion of other dementias.

The clinical diagnosis of AD is based on the criteria defined in the Diagnostic and Statistical Manual of Mental Disorders (DSM-III-R and DSM-IV, American Psychiatric Association 1987 and 1995) and on the criteria of the National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer's Disease and Related Disorders Association Work Group (NINCDS-ADRDA) (McKhann et al., 1984). According to the NINCDS-ADRDA criteria, the clinical diagnosis of AD is classified as *probable* or *possible*. A diagnosis of *definite* AD can be made only after the histopathological examination of brain autopsy or biopsy tissue samples. The criteria defined by the Neuropathology Task Force of the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) are usually the basis for the neuropathological diagnosis of AD (Mirra et al., 1991).

At autopsy, neuritic plaques and neurofibrillary tangles are the hallmark lesions that are observed in the brains of AD patients. Moreover, the extensive loss of synapses and the damage of certain neuronal population such as cholinergic cells of nucleus basalis are also the key features of AD (Terry and Katzman, 1983, Bowen, 1990). Neuritic plaques, which are also found to some extent in the brains of normal aged individuals, are mainly composed of the amyloid beta (A β) peptide in its insoluble, fibrillar form (Masters et al., 1985, Kang et al., 1987). Other essential constituents of neuritic plaques are dystrophic neurites, reactive astrocytes (RA), and activated microglia (AM) cells (Terry and Katzman, 1983). Furthermore, neuritic plaques have been shown to contain numerous molecules such as heparin sulfate proteoglycans, ApoE, and complement factors. In addition to neuritic plaques, non-fibrillar plaques (diffuse) primarily composed of the A β component and lacking the dystrophic neurites can be observed in AD and it has been suggested that diffuse plaques are the early stages or discrete niduses of neuritic plaques.

The formation of abnormally phosphorylated tau protein is the second hallmark of AD (Terry and Katzman, 1983). This phosphorylation process, which is also detected in other degenerative brain diseases, leads to production of the neurofibrillary tangles (NFT), which are intraneuronal inclusions made up of the paired helical filaments (PHF) of the abnormally phosphorylated tau. Neuropil threads (NT), which are short and tortuous neurites found in AD

brains, are also loaded with tau-positive PHFs. Ultrastructural examination of PHFs has described them as a pair of aligned helical ribbons of variable width (Pollanen et al., 1997). The exact reason for abnormal phosphorylation of tau is not yet clear, but the fact that normal autopsy samples also contain a high proportion of phosphorylated tau suggests that one factor contributing to the hyperphosphorylation in AD may be due to the inaccessibility of tau protein to phosphatases (Matsuo et al., 1994).

Approximately 10% of AD patients show clear autosomal dominant transmission of disease and 30% of AD cases have a positive family history of AD, but insufficient evidence of autosomal dominant transmission (van Duijn et al., 1991a). Thus, up to 60% of AD cases do not have any family history and are termed as sporadic. Fully penetrant, autosomal dominant mutations in APP, PSEN1, and PSEN2 genes are responsible for the early onset familial AD (Goate et al., 1991, Sherrington et al., 1995, Levy-Lahad et al., 1995, Rogaev et al., 1995), while the $\epsilon 4$ allele of ApoE is associated with an increased risk of early- and late onset familial and sporadic AD (Corder et al., 1993, Saunders et al., 1993, van Duijn et al., 1994, Chartier-Harlin et al., 1994) (Table 1). Screenings for additional susceptibility genes, particularly in the case of late onset AD have revealed several potential candidates associated with the disease including the butyrylcholinesterase (BChE), alpha-2-macroglobulin (A2M) and myeloperoxidase (MPO) genes (Lehmann et al., 1997, Blacker et al., 1998, Reynolds et al., 1999). However, the role of several potential risk genes in AD is controversial, as the numerous studies in different ethnic populations have frequently failed to replicate the initial association results, suggesting that there may be variations in the genetic risk factors of AD between different populations.

2.2. Amyloid beta precursor protein

2.2.1. Amyloid beta precursor protein metabolism

APP is a type 1 transmembrane protein, which has a long extracellular or luminal N-terminal domain and a short intracellular C-terminal domain (Hardy, 1997) (Figure 1). APP is ubiquitously expressed in different human tissues where it is alternatively spliced, particularly in neurons leading to the production of APP isoforms of different size (695, 751 and 770 amino acids) (Neill et al., 1994). Despite extensive research, the exact biological function of APP is currently unknown though it appears evident that it has pivotal role in the context of AD since APP comprises of the most important components found in the amyloid plaque, A β -peptide (Masters et al., 1985, Kang et al., 1987). This peptide, which is very prone to form fibrils, is released from APP after the cleavage of β and γ -secretases in all cell types expressing the APP holoprotein. A β consists of an up to 42 amino acid long peptide sequence, which extends from the transmembrane bilayer to the luminal or ectodomain of APP. Depending on the γ -cleavage site at the C-terminal part of APP, A β peptides of length either 40 (predominant isoform) or 42 (neurotoxic isoform) are produced while only 5-10% of secretory A β is the amyloidogenic A β 42 peptide. In addition to full length A β 42 (A β 1-42), this peptide also is possible of undergoing N-terminal heterogeneity, leading to truncated forms of different size (A β x-42) and which are also deposited in the AD plaque.

Recent data indicates that 42 and 40 isoforms of A β are produced in different intracellular compartments (endoplasmic reticulum (ER) and trans Golgi network (TGN), respectively) and the production of intracellular A β is restricted to neurons since non-neuronal cells produce A β only at the cell surface (Hartmann et al., 1997). The localization of the production of A β 42 to the ER is in concordance with the concept that PSEN 1 and 2 mutants enhance the

production of A β 42 and that the primal location of these two proteins is also in the ER. Collectively, these findings suggest that the generation of A β 42 in the ER could be the earliest event in the pathogenesis of AD.

A third protease, named α -secretase, is also involved in the processing of the APP and together with γ -secretase it produces the non-amyloidogenic peptide, p3 (Hardy, 1997) (Figure 1). In this particular process, the α -secretase cleavage site is located in the middle of the A β domain therefore preventing the production of this peptide. Thus, p3 is considered as a benign form of amyloid, since it lacks domains associated with activation and recruitment of glial cells to the neuritic plaque. While it appears that there exist several different proteases with different activities in the α , β and γ -cleavage processes, the identification of these important factors have revealed potential candidates only very recently, including the beta-site APP-cleaving enzymes 1 and 2 (BACE1 and BACE2) (Vassar et al., 1999, Yan et al., 1999, Acquati et al., 2000, Solans et al., 2000).

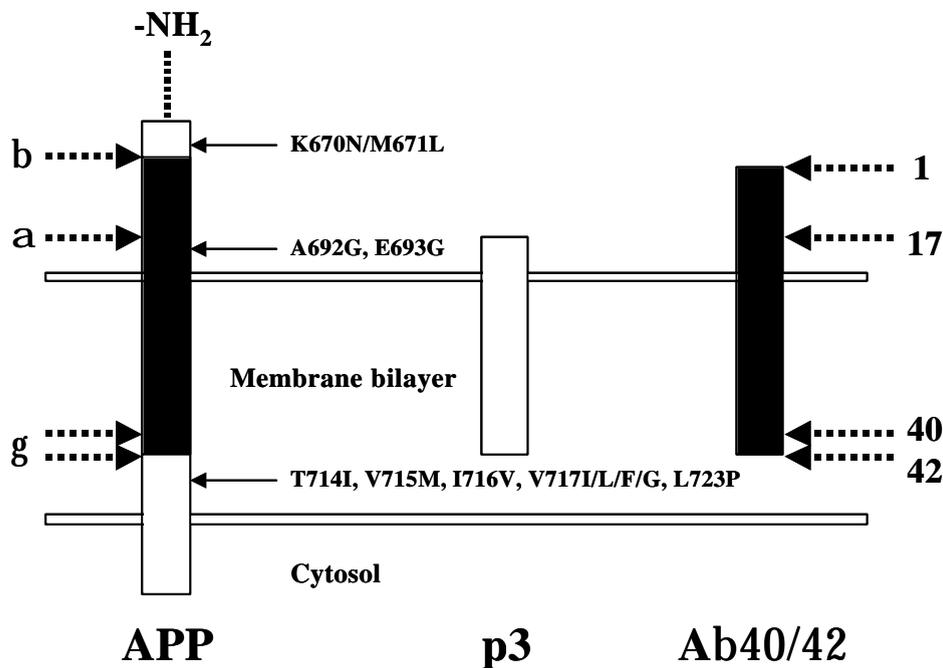


Figure 1. Domain structures of APP, p3 and A β , and secretase cleavage sites in APP. Depending on the cleavage site of γ -secretase, either 40 or 42 amino acid long A β -peptide is produced. α -Secretase produces together with γ -secretase a non-amyloidogenic peptide, p3. The positions of APP mutations causing the AD are indicated.

2.2.2. Amyloid beta precursor protein mutations and pathogeneity

According to the amyloid cascade hypothesis, the APP derivative A β 42 is the key factor in the pathogenesis of AD. In this respect, it is not surprising that the first causative mutation, which co-segregated in the early onset AD family in an autosomal dominant manner, was found in the APP gene (Goate et al., 1991). Currently, a total of eleven different APP mutations have been described in AD families and all of them are located at close to or within the domain coding the A β peptide (Alzheimer's Disease Mutation Database; <http://molgen-www.uia.ac.be/ADMutations>) (Figure 1). Mutation V717I, which is also referred to as the

London mutation, is the most frequent APP mutation comprising currently over 20 AD families. In addition, a mutation within the domain coding the A β peptide (E693Q) is responsible for a hereditably cerebral hemorrhage with amyloidosis of the Dutch type (HCHWA-D), which is autosomal dominant disease leading to cerebral angiopathy (Levy et al., 1990). With respect to the APP mutations, the range of disease onset age varies from 45 to 60 years while the ApoE genotype usually modify the exact onset age in AD families (Houlden et al., 1993). However, there is one exception concerning the mutation at codon 665, which has been identified in a late onset AD patient fulfilling the neuropathological criteria for AD suggesting that this alteration is either a rare mutation or a normal polymorphism, which coincidentally associates with the AD patient (Peacock et al., 1994). The latter explanation is favoured by the fact that both mutation and linkage analyses of the APP locus have failed to shown any association with late onset familial AD (van Duijn et al., 1991b).

The direct consequences of APP mutations are either increased production of both A β 40 and A β 42 peptides (Swedish double mutation; K670N/M671L) or A β 42 peptide alone leading to enhanced deposition of these peptides in the neuritic plaques (Hardy, 1997). This phenomenon is also evident in old APP23 transgenic mice expressing the mutant human APP (Swedish double mutation) in which case robust plaque deposition accompanied with neuronal loss has been observed throughout the entire neocortex and hippocampus (Calhoun et al., 1998). In the case of Swedish double mutation, β -secretase mediated cleavage of APP is more pronounced, probably due to fact that the substitutions in the N-terminal part of A β render the APP more susceptible for metabolism by the β pathway (Figure 1). The situation is different when mutations located at the C-terminal part of APP are considered, since these mutations alter the position of γ -cleavage favouring the enhanced production of A β 42 rather than A β 40. In this context, Flemish APP mutation (A692G) found near to the α -secretase cleavage site, leads to the inhibition of the α pathway and thus also favours the increased production of A β 42 (Hardy, 1997).

2.3. Presenilin-1

2.3.1. Presenilin-1 mutations

Mutations found in the PSEN-1 gene are a more frequent cause of autosomal dominant early onset AD than the mutations in APP and PSEN-2 genes accounting for 18-50% of the early onset AD families (Cruts and Van Broeckhoven, 1998). Since the initial characterization of the PSEN-1 locus on chromosome 14 (Sherrington et al., 1995, Alzheimer's Disease Collaborative group, 1995), approximately 80 different mutations in this particular gene comprising of 10 coding exons have been documented in early onset AD families of various ethnic origins (Alzheimer's Disease Mutation Database <http://molgen-www.uia.ac.be/ADMutations>). With the exceptions of splice-site mutations, which result in inframe skipping of exon 9 (Δ 9) from the PSEN-1 transcript (Perez-Tur et al., 1995, Kwok et al., 1997, Sato et al., 1998), nearly all other changes are missense mutations found throughout the coding sequence of PSEN-1 gene. The distributions of mutations are not random since most of these mutations occur at residues which are conserved between the PSEN-1 and 2 and which are located at the predicted transmembrane domains (TM). In addition, there are two major clusters of mutations in exons 5 and 8, which comprise approximately 60% of all known PSEN-1 mutations and which are also associated with an earlier mean age of onset (Figure 2).

The common pathogenic feature for all of these PSEN-1 mutations is that they are accompanied by the increased release of the amyloidogenic A β 42 peptide from the APP by a gain-of-function, which in turn leads to the abnormal accumulation of A β 42 peptide into the amyloid plaques in the brain of AD patients (Scheuner et al., 1996, Citron et al., 1997). Recently, a novel mutation was reported in the intron 4 splice site junction of the PSEN-1 gene resulting in two shortened transcripts with premature termination codons and one full-length transcript with insertion of three nucleotides (Tysoe et al., 1998, De Jonghe et al., 1999). Consistent with the other PSEN-1 mutations, however, only the full-length protein with the one extra amino acid was detected in the brain extracts and lymphoblast lysates of mutation carriers supporting the gain-of-function mechanism rather than loss-of-function in the underlying disease process.

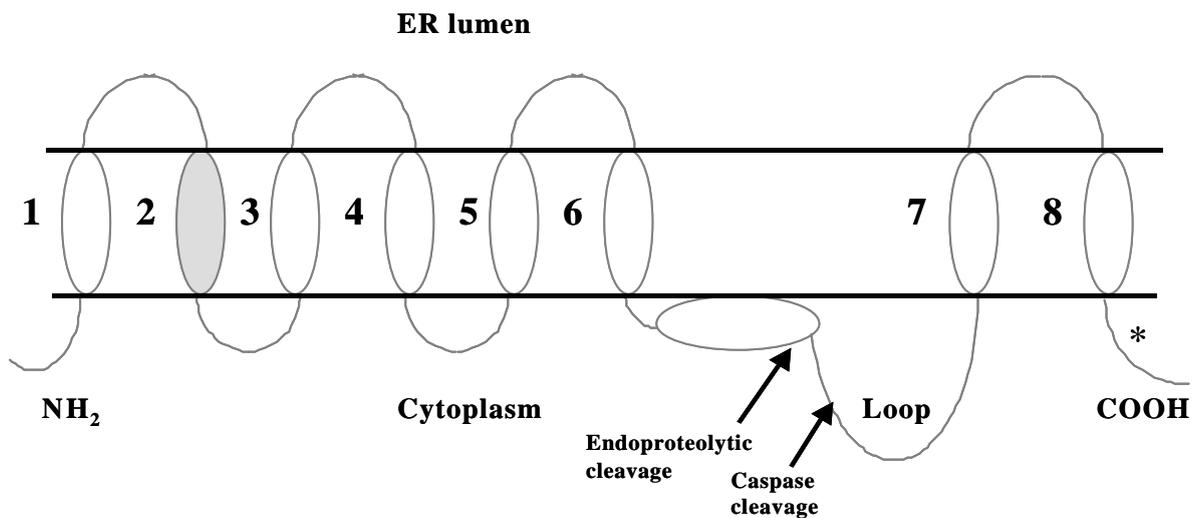


Figure 2. Transmembrane structure of PSEN-1. PSEN-1 has eight predicted transmembrane domains and the amino (NH₂) and the carboxyl (COOH) terminals reside on the cytoplasmic side of the membrane. Endoproteolytic and caspase cleavage sites are indicated. Phosphorylation sites of PSEN-1 are located in the carboxyl-terminal region (*). Transmembrane 2 and the cytoplasmic loop are the clusters of PSEN-1 mutations.

2.3.2. Cell biology of presenilin-1

PSEN-1 is expressed in most human and mouse tissues including embryonic and adult brain (Kovacs et al., 1996, Lee et al., 1996). According to *in situ* hybridization, the highest concentrations in brain are detected within the neurons of the hippocampal formation and entorhinal cortex whereas small traces of transcript are also found in white matter glial cells. PSEN-1 gene undergoes an alternative splicing event in exon 3 in which case the alternate use of the splice donor site leads to the inclusion or exclusion of codons 26-29 (VRSQ) (Alzheimer's Disease Collaborative group, 1995). In addition, exon 8 is spliced out from the PSEN-1 transcript in leukocytes due to some unknown reason (Rogaev et al., 1997).

The hydrophobic PSEN-1 protein consists of 467 amino acid residues and it is suggested to span eight times the membranes of the ER (Hardy, 1997) (Figure 2). The N- and C termini as well as the large hydrophilic loop between TM6 and TM7 are orientated towards the cytoplasmic side and are therefore available for interactions with various cytoplasmic proteins

such as β -catenin (Zhang et al., 1998). Although PSEN-1 protein is mainly located within the ER, PSEN-1 immunoreactivity is also found from the Golgi apparatus as well as plasma membranes and nuclear kinetochores. Interestingly, PSEN-1 mutations do not affect significantly the overall subcellular distribution of the protein (Kovacs et al., 1996).

The large hydrophilic loop between TM6 and TM7 encompasses the site for endoproteolytic processing of PSEN-1 protein between amino acids 291 and 292 producing ~17 kDa C-terminal (CTF) and ~28 kDa N-terminal fragments (NTF), which are controlled in a 1:1 stoichiometry (Figure 2). Subsequently after biosynthesis, PSEN-1 holoprotein is cleaved and the CTF and NTF fragments form a biologically active heterodimeric complex, which in turn is able to associate with other proteins. With the respect to $\Delta 9$ mutation, the lack of the cleavage site results in the accumulation of uncleaved PSEN-1 protein, but the abolishment of proteolytic processing is not the cause for the enhancement of pathological functions seen with this naturally occurring mutation (Steiner et al., 1999a).

2.3.3. Functions of the presenilin-1

While the exact role of the PSEN-1 is still under consideration, several lines of evidence indicate that the functions of PSEN-1 are related to the processing and trafficking of membrane proteins and apoptosis. It is obvious that PSEN-1 protein is involved in the APP processing, as both *in vitro* and *in vivo* data have revealed increased production and deposition of the amyloidogenic A β 42 peptide as a consequence of the PSEN-1 gene mutations (Scheuner et al., 1996, Citron et al., 1997). The data obtained from PSEN-1 deficient neurons also support the direct role of PSEN-1 in γ -secretase processing of APP due to the fact that the secretion of A β 40/42 and p3-peptides are strongly inhibited in these cells (De Strooper et al., 1998). In agreement with these findings, mutations produced in the intramembranous aspartate residues in TM6 and TM7 of PSEN-1, significantly reduce the γ -secretase mediated cleavage of APP as well as the normal endoproteolysis of PSEN-1 protein (Wolfe et al., 1999). Collectively, these data suggest that PSEN-1 is either the γ -secretase itself or it is a crucial cofactor protein for the actual γ -secretase. Recent data favor the latter option, as the PSEN-1 is suggested to be an essential part of the macromolecular complex, which catalyzes γ -secretase activity rather than being the protease itself (Li et al., 2000).

On the basis of work mainly conducted with the *Caenorhabditis elegans* homologue of PSEN-1, SEL-12, and with PSEN-1 knockout mice, it has been suggested that PSEN-1 may possess a more general role in the processing and trafficking of other membrane-bound proteins in addition to APP, including the Notch receptor, which is involved in cell-fate selection throughout the development (Levitan and Greenwald, 1995, Wong et al., 1997). Similarly as in APP processing, PSEN-1 protein deficiency and the aspartate mutations in TM6 and TM7 of PSEN-1 gene block the endoproteolytic cleavage of Notch, which resembles the APP cleavage mediated by the γ -secretase within the membrane. Notch intracellular cytoplasmic domain (NICD), which is released from the Notch after endoproteolysis, translocates to the nucleus, where it is needed for the signaling through selected transcription factors regulating the cell-fate selection during the development (Schroeter et al., 1998). The results obtained with the PSEN-1 and Notch knockout mice also point to a functional role for PSEN-1 in Notch signaling due to the fact that phenotypes of these knockout animals resemble each other to some extent (Herreman et al., 1999). Although the above-mentioned data are evidence for an integrated function for PSEN-1 in APP and Notch processing, mutations produced at the position 286 of PSEN-1 gene have indicated

separated biological functions for PSEN-1 in the endoproteolysis of APP and Notch (Kulic et al., 2000).

The involvement of PSEN-1 in the regulation of apoptosis has been supported by the fact that the PSEN-1 mutations in the neural cells increase the susceptibility to apoptosis induced by different apoptotic compounds (Guo et al., 1997, Tani et al., 2000). On the other hand, increased sensitivity to apoptosis due to the PSEN-1 mutations has been considered to be related to the reduced ability of PSEN-1 to stabilize the β -catenin, which in turn could predispose individuals to early onset AD (Zhang et al., 1998). Consistent with this suggestion, β -catenin levels are indeed reduced in the brains of AD patients carrying the PSEN-1 mutation. However, contradictory results concerning the proapoptotic functions of PSEN-1 mutations have also emerged since no signs of apoptosis were found in neurons, which were infected with either wild type or mutated PSEN-1 gene constructs raising the possibility of cell specific differences in the proapoptotic events (Bursztajn et al., 1998).

2.4. Presenilin-2

PSEN-2, which displays 67% homology with the PSEN-1, is also involved in the familial AD (Levy-Lahad et al., 1995). On the contrary to PSEN-1, only six missense mutations (R62H, T122P, N141I, V148I, M239V and M239I) have been so far found in PSEN-2 gene in 12 AD families and the onset age is usually later and more variable in these families than with PSEN-1 mutations. The variable expression of the disease is also evident in the novel M239I mutation family, so that some of the individuals carrying the mutations have remained unaffected beyond the onset age range (44-58 years) (Finckh et al., 2000). Also, some individuals carrying the N141I mutation in the Volga German families have remained healthy indicating an incomplete disease penetrance for certain PSEN-2 mutations.

Similar to PSEN-1, PSEN-2 is also expressed in a wide variety of human tissues and it is primarily located at the ER and Golgi complex (Kovacs et al., 1996). Although PSEN-2 holoprotein can be highly phosphorylated when compared to PSEN-1, the endoproteolytic processing of PSEN-2 also produce NTF and CTF counterparts as in the case of PSEN-1. Alternative splicing of the PSEN-2 transcript leads to the production of N-terminal truncated proteins lacking either exons 3 and 4 or exons 3, 4 and 8 (Grunberg et al., 1998). Recently, it was also demonstrated an unusual alternative splicing of the PSEN-2 gene that leads to the generation of mRNA transcript lacking exon 5 in human brain tissue (Sato et al., 1999). The appearance of this novel transcript was increased in sporadic AD patients when compared to the age-matched controls. According to the *in vitro* studies in neuroblastoma cells, the exon 5 deleted splice variant was generated in hypoxia, but not in other forms of cellular stress.

PSEN-2 mutations affect the maturation of APP protein by favouring the overproduction of A β 42 peptide both *in vitro* and *in vivo* (Mann et al., 1997, Marambaud et al., 1998). Consistent with the studies conducted with PSEN-1 mutations in the conserved aspartate residues, the corresponding intramembranous aspartates in PSEN-2 were also shown to be crucial for γ -secretase mediated APP cleavage because of the accumulation of APP-derived γ -secretase substrates and the loss of secretion of A β and p3-peptides (Steiner et al., 1999b, Kimberly et al., 2000). Moreover, mutations in the aspartate residues of PSEN-2 also block the endoproteolytic processing of the protein as well as the Notch signalling by inhibiting the release of NICD. These results indicate similar functions for both PSEN-1 and 2 in the processing of different transmembrane proteins.

The role of PSEN-2 in apoptosis is supported by the fact that the PSEN-2 can trigger pertussis toxin sensitive apoptosis (Wolozin et al., 1996) and that the N141I mutant potentiates the proapoptotic phenotype of PSEN-2 (Janicki et al., 1997). Subsequently, the increased caspase-mediated cleavage of PSEN-2 mutant protein (N141I) was found to be involved in the potentiation of apoptosis (Kim et al., 1997). Although PSEN-1 and 2 share similar functions in several processes, it appears, however, that these proteins have distinct roles in controlling the apoptosis.

2.5. Susceptibility genes involved in AD

2.5.1. Apolipoprotein E gene (ApoE)

ApoE is a polymorphic protein, which exists in three common isoforms (E2, E3, and E4) encoded by three alleles ($\epsilon 2$, $\epsilon 3$, and $\epsilon 4$) of a single gene on chromosome 19q13.2 locus (Emi et al., 1988). These allelic forms of ApoE correspond to mutations in the coding sequence of the gene resulting in amino acid substitutions (Cys and Arg) at positions 112 and 158 of the protein. While the APP and PSEN 1 and 2 gene mutations are responsible for early onset autosomal dominant AD, the $\epsilon 4$ allele of ApoE gene is associated with an increased risk of early- and late onset familial and sporadic AD (Corder et al., 1993, Saunders et al., 1993, van Duijn et al., 1994, Chartier-Harlin et al., 1994, Rao et al., 1996). It is a well-established fact that the presence of the ApoE $\epsilon 4$ allele increases the risk of AD in an allele dose-dependent manner and that this effect is also accompanied with earlier onset age (Corder et al., 1993). It is also worth noting that although the ApoE $\epsilon 4$ effect is evident in all ages between 40 and 90 years, it diminishes after the age of 70 years and that women are more likely to develop AD than men with a given ApoE genotype (Farrer et al., 1997). To date, an association of the ApoE $\epsilon 4$ allele with AD has been observed in most ethnic groups and therefore ApoE is considered to be the major susceptibility factor in AD. Conversely, bearing the $\epsilon 2$ allele of ApoE has a protective effect against AD (Corder et al., 1994, Talbot et al., 1994).

ApoE has a key role in the lipid transport since it is a constituent of several classes of plasma lipoproteins as well as being the ligand that mediates the uptake of lipoprotein particles into cells via the low density lipoprotein receptor (LDLR) and LDLR-related protein (LRP) (Mahley et al., 1988). Furthermore, ApoE is involved in the mobilization and transport of cholesterol during neuronal growth and after neuronal injury (Mahley et al., 1988, Poirier et al., 1991). In the AD brain, ApoE immunoreactivity is detected in senile plaques, neurofibrillary tangles, and cerebrovascular amyloid (Namba et al., 1991). However, the biological mechanisms by which different ApoE isoforms can mediate their effects in the neuropathogenesis of AD are not completely understood. It has been reported that the ApoE E4 isoform would facilitate the deposition of A β or affect the clearance of this peptide, since an increased number of plaques have been found in the cerebral cortex of ApoE $\epsilon 4$ homozygotes (Schmechel et al., 1993). Furthermore, ApoE2 and E3 isoforms, but not the ApoE4 isoform, have been shown to prevent the aggregation of tau protein and the formation of paired helical filaments by limiting the abnormal phosphorylation of tau protein (Strittmatter et al., 1994). Interestingly, ApoE isoforms appear to have different abilities also in preventing oxidative stress since the ApoE4 isoform has revealed decreased antioxidant activity *in vitro* when compared to ApoE2 and E3 isoforms (Miyata and Smith, 1996).

Until recently, there has been little data concerning the expression of ApoE in AD whereas the available data from other sources have indicated that the expression of ApoE is upregulated in astrocytes after neuronal damage suggesting that quantitative changes in

expression of this protein are part of a regeneration process (Poirier et al., 1994). Recently, it was suggested that other genetic variants within the ApoE gene can modify the risk of AD by altering the level of allelic-specific ApoE expression as the relative mRNA expression of ApoE ϵ 4 allele was increased in AD patients when compared to controls (Lambert et al., 1997).

2.5.2. ApoE gene promoter polymorphisms

Sequence analyses of the ApoE promoter region have revealed several polymorphisms (-491A/T, -427T/C, and Th1/E47csG/T (-219G/T)), which have been shown to modify the risk of AD in different populations (Artiga et al., 1998a, Lambert et al., 1998a). Assessment of the -491A/T polymorphism among the Spanish late onset AD patients and control subjects has revealed that the -491AA genotype increases the risk of AD independently of the ApoE ϵ 4 allele status (Bullido et al., 1998). According to *in vitro* studies, the -491A allele influences the risk of AD through the modulation of transcriptional activity of the ApoE gene since the A allele possesses higher constitutive ApoE expression than the T allele. On the other hand, changes in transcription levels may be explained on the basis of data obtained from electrophoretic mobility shifts assays in which differential binding of the nuclear proteins to the allele-specific ApoE promoter sequences has been observed.

Similarly, the allelic variant of -427C and the haplotype -491A/-427C have been found to confer increased risk of AD among the Spanish ApoE ϵ 4 non-carriers (Artiga et al., 1998b). Increased transcriptional activity of the promoter encompassing the haplotype -491A/-427C relative to the promoters of haplotypes -491T/-427C and -491T/-427T was detected in human astrocytoma cells. In line with suggestions concerning the altered expression of ApoE due to the promoter polymorphisms, the -491AA genotype has been shown to associate with increased plasma ApoE levels in AD patients, providing the basis for evaluating the effects of this polymorphism *in vivo* (Laws et al., 1999). Lambert et al., (1998b) have also shown in their epidemiological study that the T allele of Th1/E47cs polymorphism is associated with increased risk of AD independently of the ApoE allele ϵ 4 status. According to *in vivo* studies, Th1/E47cs polymorphism also affects the expression levels of ApoE since the presence of the T allele increases the relative expression of ApoE ϵ 4 mRNA in AD brain samples, but not in control samples carrying the corresponding polymorphisms in the promoter and coding regions.

Collectively, these results suggest that increased expression of ApoE due to the different promoter polymorphisms in conjunction with qualitative modifications (ApoE ϵ 2/3/4) could be an important determinant in AD. Therefore, variations in the ApoE expression status induced by individual promoter polymorphisms or their combinations may also explain the heterogeneity in the impact of the ApoE ϵ 4 allele in different ethnic populations. However, it is still unclear whether these effects are independent of the ApoE ϵ 4 allele since studies conducted with different ethnic populations have indicated linkage disequilibrium between promoter polymorphisms and the ApoE ϵ 4 allele, which in turn could explain most of the promoter polymorphism associations with AD (Town et al., 1998, Roks et al., 1998, Rebeck et al., 1999, Thome et al., 1999, Zurutuza et al., 2000).

2.5.3. Butyrylcholinesterase K variant gene

BChE is a hydrolytic enzyme, which is expressed in most human tissues including certain regions of the brain such as the hippocampal formation and amygdala (Darvesh et al., 1998).

Although the exact physiological function of BChE is still unknown, it has been considered to possess related functions with the acetylcholinesterase (AChE) in the nervous system as a coregulator of the action of acetylcholine (ACh) suggesting that BChE may play a role in cholinergic neurotransmission (Atack et al., 1986, Giacobini et al., 1996). In addition, BChE shares features *in vitro* with other cholinesterases in regulating the neurite growth and cell adhesion by means of non-enzymatic functions (Layer et al., 1993). The fact that BChE expression is substantially increased in AD brains (Perry et al. 1978) and that BChE is associated with the neurofibrillary tangles and amyloid plaques (Gómez-Ramos et al., 1994) point to a possible role for this enzyme also in the neuropathogenesis of AD. Consistent with this idea, BChE was recently shown to participate in the A β transformation process changing A β from a benign to an eventually malignant form (Guillozet et al., 1997).

There are at least seven different genetic variants of BChE and the K variant is suggested to be the most common alteration (G \rightarrow A at the position 1615) accounting for 12-15% allele frequency in the Caucasian population (Bartels et al., 1992, Jensen et al., 1996). This single nucleotide change at the BChE K variant gene leads to an amino acid substitution of alanine to threonine at codon 539 accompanied by a 30% reduction in enzymatic activity. Interestingly, Lehmann et al., (1997) reported that the frequency of BChE K allele was significantly increased in late onset AD patients carrying the ApoE ϵ 4 allele suggesting that these two alleles act in synergy in AD. This effect was more pronounced among the AD patients group in which the age of onset was over 75 years, giving an odds ratio of 12.8 for those patients carrying both the BChE K and ApoE ϵ 4 alleles. The follow-up studies concerning the BChE K variant have revealed controversial results in the context of AD. The majority of these studies show no association of the K variant allele either alone or in combination with the ApoE ϵ 4 allele (Brindle et al., 1998, Singleton et al., 1998, Russ et al., 1998, Crawford et al., 1998, Kehoe et al., 1998, Ki et al., 1999, Grupper et al., 1999, Yamamoto et al., 1999). However, there are also findings which support an increased risk for BChE K variant and ApoE ϵ 4 alleles particularly among very old AD patients (onset age \geq 75 years) suggesting that BChE K variant is a reasonable candidate for influencing the course of AD with increasing age (Wiebusch et al., 1999, Tilley et al., 1999, McIlroy et al., 2000).

The differences between studies concerning the BChE K allele polymorphism in AD may originate from several possible sources. It is possible that the BChE K variant allele has a different effect in the genetically different subsets of population, which therefore affects the risk of AD in a context-dependent manner. This explanation is supported by the fact that in two of these studies, including the initial one, AD and control subjects were collected from England suggesting a common genetic origin for those two study cohorts (Lehmann et al., 1997, Tilley et al., 1999). Alternatively, it is possible that another susceptibility gene nearby to the BChE gene locus on chromosome 3 is in linkage disequilibrium with the K variant allele. This option, however, is not supported by the data obtained with markers within the BChE gene, suggesting that BChE acts through its K variant rather than a nearby susceptibility gene (Wiebusch et al., 1999). In line with this suggestion, association of the BChE K variant allele with cholinesterase-positive neuritic plaques in the temporal cortex was observed in late onset AD patients (Lehmann et al., 2000). This observation also suggests that the K variant itself could be involved in AD pathogenesis in promoting fibril formation or induction of aberrant neurite growth.

2.6. Mapping for novel AD genes

Mapping for novel late onset AD genetic loci using conventional family-based linkage analyses encounters problems, which are related to the complex inheritance of the disease as well as the limited number of affected members in different generations available for genotype and phenotype analyses. These factors significantly reduce the power to detect susceptibility genes with small effects (relative risk less than 4) and therefore alternative approaches such as association-based (Risch and Merikangas, 1996) and multipoint oligogenic analyses (Daw et al., 1999) have been proposed for localizing novel genetic loci.

In recent years, a few large genome-wide screens have been performed for finding additional genetic risk factors for late onset AD using both model-dependent as well as model-independent methods (Pericak-Vance et al., 1997, Zubenko et al., 1998, Kehoe et al., 1999) (Table 1). According to the complete genomic screen conducted by Pericak-Vance et al. (1997) with multiplex families affected with late onset AD, 15 chromosomal regions were detected during the initial search, which warranted follow up analyses. Four of those regions on chromosomes 4, 6, 12, and 20 were still linked after the follow up analyses and the chromosome 12 locus indicated the most consistent multipoint maximum lod score (MLS) result. Since then, linkage result concerning the chromosome 12 locus has been confirmed in several studies (Wu et al., 1998, Rogaeva et al., 1998, Scott et al., 1999, Scott et al., 2000) though the actual susceptibility gene has remained undiscovered. A potential candidate in this region is the serum pan-protease inhibitor, A2M gene, which has been shown to be genetically and neuropathologically associated with AD (Blacker et al., 1998, Liao et al., 1998, Myllykangas et al., 1999). However, several lines of evidence suggest that some other nearby gene on that particular chromosome region could be the main determinant rather than A2M itself.

In a full genome scan for late onset AD using affected sibling pairs (ASPs), Kehoe et al. (1999) suggested that several other risk gene loci exist in addition to ApoE gene locus and the highest lod scores fulfilling the definition of suggestive linkage were found on chromosomes 1, 9, 10, and 19. Consistent with these findings, quantitative-trait loci (QTLs) analysis using plasma A β levels as the surrogate marker in late onset AD pedigrees yielded lod scores with suggestive evidence for linkage with the two above-mentioned chromosome loci (Taner et al., 2000).

Chromosome locus	Gene or marker	Approach	Reference
1p13-q12	D1S1675-D1S1595	Linkage	Kehoe et al., 1999
1q41	PSEN 2	Linkage/ Candidate gene	Levy-Lahad et al., 1995
3q26	BChE*	Candidate gene	Lehmann et al., 1997
4q28-q31	D4S1629-D4S2368	Linkage	Pericak-Vance et al., 1997
6p21	D6S1019*	Linkage	Pericak-Vance et al., 1997 Garcia et al., 1999
9q22-q33	D9S176-D9S279	Linkage	Kehoe et al., 1999
10p13	D10S1423*	Association	Zubenko et al., 1998 Majores et al., 2000
10p12-q22	D10S1426-D10S675	Linkage	Kehoe et al., 1999
12p13	A2M	Candidate gene	Blacker et al., 1998
12p12-q13	D12S373-D12S390	Linkage	Pericak-Vance et al., 1997 Wu et al., 1998 Rogaeva et al., 1998 Scott et al., 1999 and 2000
14q24	PSEN 1*	Linkage	Sherrinton et al., 1995 Alzheimer's Disease Collaborative group, 1995
17q21	MPO	Candidate gene	Reynolds et al., 1999
19q13	ApoE*	Linkage/ Association	Pericak-Vance et al., 1991 Corder et al., 1993 Saunders et al., 1993
19q13	D19S412-D19S571	Linkage	Kehoe et al., 1999
20q13	D20S94	Linkage	Pericak-Vance et al., 1997
21q22	APP	Linkage	St George-Hyslop et al., 1987

Table 1. Genes and chromosome regions found to be linked to AD.

* Genes and chromosome regions found to be linked to AD in the present study.

This result suggests that genetic susceptibility factors also influence the plasma A β levels in late onset AD, reinforcing the use of QTL mapping as an alternative tool for finding novel AD-associated gene loci. Daw et al. (2000) have also estimated the number and effects of additional QTLs on age at onset of AD by performing oligogenic segregation analysis. In addition to the ApoE locus, which was estimated to have a 7 to 9% contribution to the total variation in onset of AD, four additional QTLs were suggested to exist, with one of these loci possessing even a greater impact than the ApoE.

2.6.1. Linkage disequilibrium mapping

Linkage disequilibrium (LD) mapping of disease genes is based on assumption that a single ancestral mutation is responsible for a large proportion of disease cases found in the present day population and the chromosome on which the mutation originally was introduced carries a particular set of marker alleles, which are forming an ancestral founder haplotype (Jorde, 1995) (Figure 3). Consequently, the disease allele (mutation) can be detected only in the

presence of a specific set of original marker alleles, which are identical-by-descent (IBD) and in complete LD with the disease allele. After subsequent generations, recombinations occur between the disease allele and the surrounding marker alleles dissipating gradually the disequilibrium. With this respect, marker alleles located closest to the disease allele will display higher disequilibrium than the marker alleles located more distantly.

Traditionally LD mapping has been used successfully for fine mapping and cloning of monogenic disease genes, which have been already localized to a particular chromosomal region using conventional linkage analyses (Hästbacka et al., 1994, Höglund et al., 1995). Also, LD-based methods such as case-control association or family-based transmission disequilibrium tests have been frequently used in candidate gene studies of different diseases. In contrast, genome-wide LD mapping has been limited to very rare cases mainly due to the fact that in heterogenous populations, chromosome regions displaying the LD are expected to be quite small (1-2 cM). Recently, Zubenko et al. (1998) reported a genome survey for late onset AD in which they have used microsatellite markers spaced on average 10 cM distance from each other. Altogether six markers were found to be associated with AD including the marker D19S178, which is located approximately 2.5 cM from the ApoE locus suggesting that this currently already known risk gene locus could have been found after the subsequent studies.

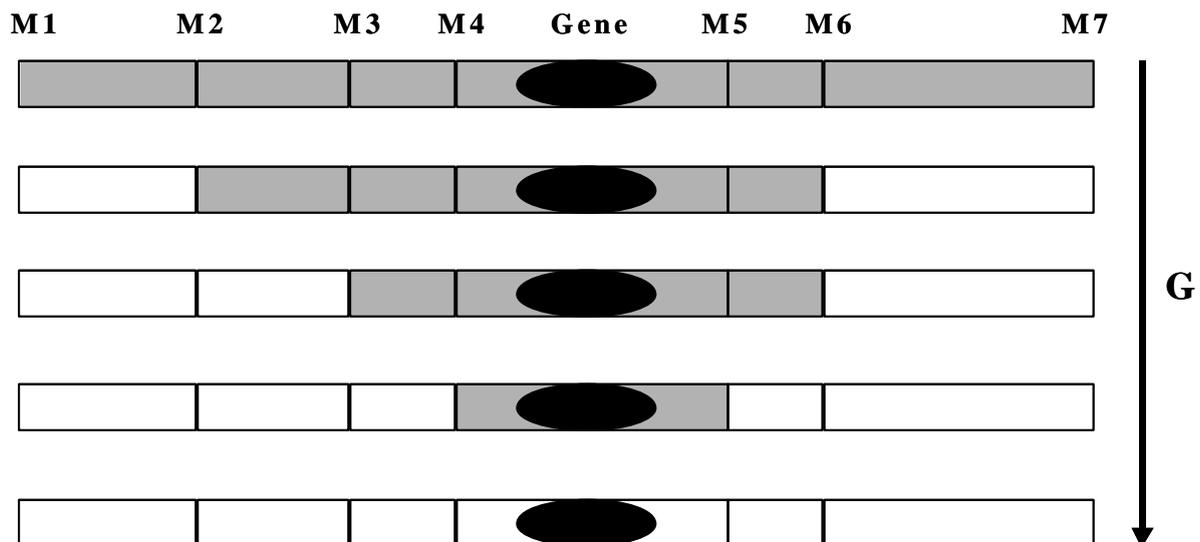


Figure 3. Principal of linkage disequilibrium. A mutation has occurred in the gene of the ancestral chromosome (top). In a series of generations (G), recombinations occur between disease allele and the surrounding marker (M) alleles dissipating gradually the disequilibrium (gray color). Marker alleles, which are located in the close vicinity of disease allele, encompass stronger linkage disequilibrium than marker alleles located more distantly.

2.6.2. Factors influencing the linkage disequilibrium

In general, the key factors influencing the detection of LD are the recombination frequency and the number of generations since the mutation was introduced into the population (Jorde, 1995, Wright et al., 1999). The effect of marker allele age is evident in the case of ApoE gene as the SNP studies in the vicinity of this gene locus reveal evidence of an association with AD only with markers spanning 40 kb on either side of ApoE (Martin et al., 2000). This result is

consistent with the estimation data in which the calculated distance between ApoE ϵ 4 locus and nearby marker loci displaying LD was suggested to be only 35 ± 5 kb reflecting the evolutionary ancient nature of ApoE ϵ 4 allele, which originates before the divergence of the present day populations 100 000 years ago (Gong et al., 1999).

In addition, factors such as genetic drift, expansion of the population, migration, and inbreeding also influence the power of detecting LD for which reason the choice of the population in LD mapping of complex disease genes is considered to be a critical issue (Wright et al., 1999). Recent genetic founder populations (< 200 generations) with reduced number of the original founders may then allow the detection of LD between disease and marker loci over a large chromosomal interval when compared to more admixed, cosmopolitan populations. This effect is particularly evident in Finland in which regional population bottlenecks have created clusters of several Finnish heritage diseases and the carrier frequencies of these disease mutations reveal a distinct geographic occurrence (de la Chapelle 1993, Peltonen et al., 1999, Pastinen et al., 1999). As consequence of this, LD regions of over 10 cM have been detected in the case of rare Mendelian diseases such as congenital chloride diarrhoea, Northern epilepsy, and Salla disease (Peltonen et al., 1999). The situation with common diseases, however, could be different since the initial number of founders carrying the disease has been probably larger than with the rare diseases resulting in several disease-associated alleles and thus affecting the detection of IBD alleles (Kruglyak, 1999). Nevertheless, recent data obtained from genome-wide screen of schizophrenia indicate that descendents derived from sub-populations founded in the late settlement regions in Finland during the 16th and 17th centuries may provide a suitable population also for low-density LD mapping studies of complex diseases (Hovatta et al., 1999).

3. AIMS OF THE STUDY

This study is a part of an ongoing molecular genetic study in which the aim is to assess genetic components involved in early and late onset AD in Finland. Despite the well-established effects of the APP, PSEN-1, PSEN-2 and ApoE genes in early and late onset AD, it is evident that additional susceptibility genes are involved in the underlying disease process. In order to locate these novel gene loci associated with AD, genome-wide linkage disequilibrium mapping was conducted using Eastern Finnish late onset AD patients and age-matched controls. In addition, the effects of different susceptibility and causative genes among the Finnish early and late onset AD patients were examined.

The specific aims of the study were:

- 1) To determine the genetic cause in an early onset AD family in which the transmission of the disease indicated clear autosomal dominant inheritance by screening the PSEN-1 gene mutations (**Study I**).
- 2) To study the role of substitution E318G in the PSEN-1 gene in familial and sporadic AD populations (**Study II**).
- 3) To determine whether the BChE K variant and ApoE ϵ 4 genes are acting in synergy in late onset AD patients (**Study III**).
- 4) To study ApoE gene promoter -491A/T polymorphism and its dependence on the ApoE ϵ 4 allele in late onset AD patients (**Study IV**).
- 5) To conduct a genome-wide linkage disequilibrium mapping for finding novel AD-associated chromosomal loci using Eastern Finnish late onset AD patients and age-matched control subjects from a geographically restricted area in Eastern Finland (**Studies V/VI**).

4. SUBJECTS, MATERIALS AND METHODS

4.1. Subjects

This study was performed during 1997-2000. AD patients and control subjects originating from Eastern Finland were examined at the Kuopio University Hospital (Studies II-VI). Familial early onset AD patients used in the study I were clinically examined in Helsinki University Hospital whereas the neuropathological study was carried out in Kuopio University Hospital. This study was approved by the Ethics Committees of Kuopio and Helsinki University Hospitals. Demographic data concerning the AD patients and control subjects used in studies II-VI are indicated in Table 2.

Study I

The family examined included four affected members with early onset dementia (the mean onset age and the mean duration of the disease was 43 ± 2 and 4 ± 2 years, respectively) and the transmission of the disease indicated autosomal dominant inheritance (see AD pedigree in Figure 4). Neurological examinations, neuropsychological tests, CT scanning and differential diagnostic laboratory tests were performed for three affected individuals (II:2, II:4 and II:5). For patient II:2, EEG, SPECT, and psychiatric evaluation were made and the patient was diagnosed as having probable AD. Brain biopsy from the cortical region was taken from patient II:4 and the definite AD was diagnosed according to the CERAD criteria (Mirra et al., 1991). Patients II:2, II:4 and II:5 were regularly examined by neurologists after the disease onset. Patient I:2, who had died long before the present study, was clinically examined in the 1970's and the patient was diagnosed as demented. In addition, 51 neurologically examined control subjects and 102 familial and sporadic early- and late onset AD patients from Eastern Finland, who fulfilled the NINCDS-ADRDA criteria for definite, probable, or possible AD (McKhann et al., 1984) were used for mutation screening.

Study II

Substitution E318E in the PSEN-1 gene was screened from sixteen unrelated familial AD patients, who were clinically diagnosed as probable AD according to the NINCDS-ADRDA criteria (McKhann et al., 1984) (Table 2). In addition, 64 sporadic neuropathologically confirmed AD patients (Mirra et al., 1991) as well as 270 clinically and neuropathologically examined control subjects were screened for the substitution.

Table 2. Demographic data of the AD patients and controls used in the studies II-VI.

	Groups	Number	Age ^a	Age range	Male/Female
Study II	Familial AD		72 ± 7	50-78	5/11
	EOAD	1			
	LOAD	15			
	Sporadic AD	64	72 ± 9	45-90	10/54
	Controls	270	76 ± 3	47-98	93/177
Study III	Clinical	235			
	Confirmed	35			
	LOAD	78	73 ± 5	65-89	20/58
	Clinical	61			
Study IV	Confirmed	17			
	Controls	97	72 ± 4	65-86	33/64
	LOAD	106	73 ± 6	59-89	29/77
Study V	Controls	123	73 ± 4	64-87	47/76
	LOAD	47	75 ± 6	59-84	18/29
Studies V/VI	Familial	20			
	Sporadic	25			
	Controls	51	70 ± 5	64-86	17/34

^a Mean age at onset for AD patients and mean age at time of neurological evaluation or at time of death for controls. EOAD = Early onset AD, LOAD = Late onset AD.

Study III

Interaction between BChE K variant and ApoE ε4 alleles was studied in a group of subjects ≥65 years of age consisting of 78 late onset AD patients and 97 non-demented control subjects (Table 2). The AD group consisted of both possible (17 %) and probable (61 %) AD patients according to the NINCDS-ADRDA criteria (McKhann et al., 1984) as well as neuropathologically confirmed definite AD patients (22 %) (Mirra 1991).

Study IV

ApoE promoter polymorphism (-491A/T) was genotyped from 106 late onset AD patients, who clinically fulfilled the diagnosis of probable or possible AD (McKhann et al., 1984) and from 123 community-based control subjects without any signs of cognitive impairment (Table 2). The gender and age distributions in AD patients did not differ significantly from those in controls.

Studies V/VI

Forty-seven late onset AD patients, who fulfilled the NINCDS-ADRDA criteria for possible or probable AD (McKhann et al., 1984) with mean Mini Mental Status Examination (MMSE) of 18.6 ± 4.6 points (Folstein et al., 1975) and 51 unrelated voluntary controls with MMSE ≥ 27 points were examined at Kuopio University Hospital (Table 2). The AD patients and controls were derived from a restricted area of eight communities in the province of Northern Savo in Eastern Finland. The area was settled in the late 16th and early 17th century

(Soininen 1981, Pirinen 1982). Controls were selected from a large group of subjects who responded voluntarily to advertisements seeking non-demented control subjects of ≥ 65 years of age originating from the above-mentioned communities. To confirm the homogeneity of the study groups, the birthplace of the patients, controls and their parents were recorded. AD patients and controls did not differ significantly in age or gender distributions. The AD group consisted of patients both with positive familial history of AD, but inconclusive evidence of autosomal dominant transmission and patients having sporadic AD. Patients were considered as familial cases if at least two first-degree relatives with dementia in two different generations were documented (Lehtovirta et al., 1996). Familial patients were unrelated and for two patients there was no reliable family history available.

4.2. Gene analyses

4.2.1. DNA and RNA extractions

Genomic DNA was extracted from peripheral lymphocytes using a phenol-chloroform-isoamyl alcohol method (Vandenplas et al., 1984) and from paraffin-embedded tissues in postmortem AD cases as described by Isola et al. (1994). Total RNA was extracted from peripheral lymphocytes and from postmortem temporal cortex using Trizol Reagent (Gibco BRL). Poly (A⁺) mRNA was captured using mRNA Capture Kit (Boehringer Mannheim).

4.2.2. Polymerase chain reaction analyses

Polymerase chain reaction (PCR) primers and annealing temperatures used in the studies I-VI are indicated in Table 3. PCR primers and cycle conditions for microsatellite markers are found on the Genome Database (<http://WWW.gdb.org/>). In general, PCR analyses were carried out using standard procedures with PTC-100TM or PTC-200TM Programmable Thermal Controllers (MJ Research Inc). The total amount of genomic DNA varied from 25 to 100 ng depending on the PCR application used.

In study I, poly (A⁺) captured lymphocyte mRNA was used for PSEN-1 gene RT-PCR analysis (TitanTM One Tube RT-PCR System, Boehringer Mannheim) with exons 8 (DEL9F) and 10 (DEL9R) specific primers (Table 3 and Figure 5). Both wild-type (348 bp) and truncated (261 bp) PCR products were excised from 1% low melting agarose gel and cycle-sequenced in both directions with ABI PRISM 310 genetic analyser (see chapter 4.2.3). In study V, expression status of the H, K-ATPase α -subunit protein (ATP1A1) in the temporal cortex was determined using RT-PCR (TitanTM One Tube RT-PCR System) with the primers and conditions previously described (Table 3, Modyanov et al., 1991). The RT-PCR product was purified and cycle-sequenced in both directions.

4.2.3. Sequence analyses

The exons of open reading-frame of the PSEN-1 gene (exons 3-12) were PCR amplified with similar primers and conditions as reported by Hutton et al. (1996). PCR products were purified using QIAquickTM PCR Purification Kit (Qiagen) and cycle-sequenced with the ABI PRISM 310 genetic analyser (Perkin Elmer) and ABI Prism Sequencing 2.1.1 program (Perkin Elmer) by utilizing the dReady Dye Terminator Cycle Sequencing kit with AmpliTaq DNA polymerase, FS (Perkin Elmer).

4.2.4. Genomic DNA analyses of presenilin-1 gene

Microsatellite markers D14S77 and D14S1025, which are flanking 5' and 3' of the PSEN-1 gene, respectively, were genotyped using fluorescent-labelled PCR-primers. The sizes of PCR products were determined with ABI PRISM 310 genetic analyser and Genescan 2.1 analysis program (Perkin Elmer). Intron 8 polymorphism of PSEN-1 gene was genotyped by PCR using mismatch primers (Table 3 and Figure 5) and *Bam*HI restriction enzyme (New England Biolabs) digestions as previously described (Wragg et al., 1996). On the basis of genotype and pedigree data, extended haplotypes were reconstructed for each family member.

Identification of genomic deletion break-point region from the genomic DNA in study I was conducted using Expanded Long PCR System (Boehringer Mannheim) with introns 8 (IN8F4) and 9 (IN9F5) specific primers (Table 3 and Figure 5). Cycle sequencing was performed with a novel 1.6-kb PCR-product, which was detected only in the affected members of the family. On the basis of the data obtained from the sequence analysis, multiplex PCR primers DE8F1, DE8R2 and DE9R1 were designed for detecting control (504 bp) and deleted (290 bp) fragments (Table 3 and Figure 5). *Alu* and other repeat sequences were identified using the BLAST 2.0 program (<http://www.ncbi.nlm.nih.gov/>) and the Repeat Masker Server (<http://ftp.genome.washington.edu/>).

A substitution leading to the amino acid change from glutamic acid (E) to glycine (G) at codon 318 (E318G) of the PSEN-1 gene in study II was screened using the PCR-restriction fragment length polymorphism (PCR-RFLP) method (Dermaut et al., 1999). Mismatch PCR primer was designed to detect the alteration from adenine to guanine (GAA to GGA) in the last codon of exon 9 (Table 3 and Figure 5). Amplified PCR fragment (75 bp) was subsequently digested with *Bst*NI (New England Biolabs) for 4 h at 60°C to produce 52 and 23 bp fragments in the case of E318G substitution. After the digestion, electrophoresis was carried out on 3% agarose gel. A sequence verified heterozygote E318G sample was used as a control.

4.2.5. Determination of butyrylcholinesterase K variant allele form

To detect the BChE K variant allele form, which encompasses a point mutation G to A at the position of 1615 accompanied by an amino acid change from alanine to threonine, PCR and digestion of the PCR products were carried out as previously described (Table 3, Jensen et al., 1996). Amplified PCR fragment (137 bp) was digested with *Mae*III (New England Biolabs) overnight at 55°C. In the presence of the K allele, 115 and 22 bp DNA fragments were detected on the 2% agarose gel. DNA samples, which were verified as normal, heterozygote, or homozygote BChE-K variants by cycle sequencing, were used as controls in the allele determinations.

Table 3. PCR primers and annealing temperatures (°C) used in the genomic DNA and RT-PCR amplification analyses.

Primers ^a	
Study I (57): PSEN-1 RT-PCR	DEL9F: 5'-GCT GTT TTG TGT CCG AAA GGT CCA CTT CGT ATG CTG-3' DEL9R: 5'-CTC TGG GTC TTC ACC AGC GAG GAT ACT GCT GGA AAG-3'
Study I (60): PSEN-1 Introns 8/9	IN8F4: 5'-AAG TGG TTC ACT CTG GGA GCT TAA C-3' IN9F5: 5'-GAA AGC TCT TCC TCC ATC CTT CAG C-3'
Study I (52): PSEN-1 Multiplex-PCR	DE8F1: 5'-GTG GAC ATT GAT TTT CAT GTT AC-3' DE8R2: 5'-TCT AAT ACT CAG GCT TCT TGA AG-3' DE9R1: 5'-TAT ACC TAC ATG CTC ACA GAC AAT C-3'
Studies I/II (45): PSEN-1 Intron 8	F: 5'-CAC CCA TTT ACA AGT TT AGC-3' R: 5'-CAC TGA TTA CTA ATT CAG <u>G</u> AT C-3'
Study II (45): PSEN-1 E318G	F: 5'-TCC AAAAAT TCC AAG TAT AAT <u>CC</u> -3' R: 5'-ACA GTG ACC CTG AAA AAT CAA GA-3'
Study III (53): BChE K variant	F: 5'-ATA TTT TAC AGG AAA TAT TGA TGT <u>A</u> -3' R: 5'-ATT AGA GAC CCA CAC AAC TT-3'
Study IV (60): ApoE -491A/T I	F: 5'-CTC GAC TTT TAG CAG GTG CA-3' R: 5'-CAA GCG TGG AAG GGG AAT G-3'
Study IV (62): ApoE -491A/T II	F: 5'-TGT TGG CCA GGC TGG <u>TTT</u> TAA-3' R: 5'-TCC TTT CCT GAC CCT GTC CT-3'
Study V (48): ATP1AL1 RT-PCR	F: 5'-AGA TTC CGA GAA GAA GAC CA-3' R: 5'-GCT GGG GCT CAG ACT CCC CCG TGA GA-3'
Studies II-VI (60): ApoE	F: 5'-GCA CGG CTG TCC AAG GAG CTG CAG GC-3' R: 5'-GGC GCT CGC GGA TGG CGC TGA G-3'

^a Mismatch nucleotides are underlined.

4.2.6. Determination of ApoE -491A/T promoter polymorphism

Genotyping of the ApoE promoter -491 A/T polymorphism was conducted using nested PCR strategy (Bullido et al., 1998). DNA region spanning -784 to -213 relative to the ApoE transcription-starting site was initially PCR amplified in the 15-cycle reaction (-491A/T I primers; Table 3). Subsequently, 1 μ l of the PCR product was used as a template for a second PCR reaction with primers spanning the region -512 to -283 including also the -491 A/T polymorphism site (-491A/T II primers; Table 3). To detect the A allele at the -491 site, a 226 bp PCR product was digested with *DraI* (New England Biolabs) and the DNA fragments were visualized on 3% agarose gel. A sequence verified heterozygote -491 A/T sample was used as a control.

4.2.7. Determination of ApoE allele forms

For detection of the ApoE allele forms (ϵ 2, ϵ 3 and ϵ 4), PCR and *HhaI* (New England Biolabs) digestions of the PCR products were carried out with primers and conditions as previously described (Table 3, Tsukamoto et al., 1993). The heterozygote ApoE ϵ 2/4 sample was used as control in each run.

4.2.8. DNA analysis of microsatellite markers

In the initial genome-wide screening, 366 fluorescent-labelled microsatellite markers from the sixth version of the Weber lab screening set (see Appendix: Microsatellite markers used in the initial genome-wide screening) were PCR amplified using multiplex approach with ATG-polymerase enzyme (Perkin Elmer). Sizes of the PCR-products were determined with ABI PRISM 310 genetic analyzer and the Genescan 2.1 analysis program. The screening set included markers from the chromosomes 1-22 and X. The average spacing between markers was 10 cM and the average heterozygosity value was 0.76. Some of the CHLC markers were replaced with markers from the Généthon and Marshfield marker maps. The location and order of additional microsatellite markers (1 to 8 markers/locus) used in the second screening of the 22 LD regions were obtained from <http://cedar.genetics.soton.ac.uk/>, <http://marshmed.org/>, and <http://WWW.gdb.org/>. Sex-average or female specific genetic maps were used for estimating the distances between individual markers.

4.3. Neuropathology

In study I, a diagnostic cortical biopsy was taken in 1992 from the patient II:4 (Figure 4) and received in two pieces, each approximately 10 mm in diameter. After fixation and paraffin embedding, seven- μ m thick sections were cut and stained with Hematoxylin-Eosin, Thioflavin-S and modified Bielschowsky silver impregnation. Furthermore, the tissue was analysed applying immunohistochemical methodology using antibodies directed towards β -amyloid (β A4 -DAKO M872, dilution 1:100; β A40 and β A42 -US Peptides, 1:1500; 4G8 and 6E10 -Senetek, 1: 2000), hyperphosphorylated tau (AT8 -Innogenetics BR03, 1:500), α -synuclein (Synuclein 1 -Transduction Lab S63320, 1:1000; α -synuclein -ZYMED LB509, 1:500), glial fibrillary acidic protein (GFAP -DAKO Z0334) human histocompatibility class II antigen (HLA DR -DAKO M775) and complement components (C1q -DAKO A0136, C3c -DAKO A0062, C3d -DAKO A0063 and C5 -DAKO A0055, 1:100).

4.4. Statistical analyses

The distributions of genotypes in studies II-V were found to be in Hardy-Weinberg equilibrium (Associate program, version 2.31). The level of statistical significance was defined as $P < 0.05$.

Studies II-IV

The standard contingency-table chi-squared or Fischer's exact tests with SPSS software were used to analyse the difference between study groups and the odds ratios (OR) were calculated using 95% confidence intervals (CI). In study IV, the t test for independent samples and one-way analysis of variance followed by Duncan post hoc analysis were used to compare ages between study groups and differences in age at onset, respectively. EH-program, version 1.12 (Terwilliger and Ott, 1994) was used to estimate haplotype frequencies for ApoE and -491 A/T alleles.

Study V

The linkage disequilibrium analysis at the initial screening was performed using the CONTING program, version 2.51 (Terwilliger and Ott, 1994). Testing the significance of an individual marker allele was carried out by pooling other alleles into one group and calculating the results using the contingency table chi-squared tests with the Yates' correction. Linkage disequilibrium of the markers at 13q11-q12 was calculated using two-tailed Fisher's exact test (Monte Carlo estimate with 99% confidence level) with SPSS software, version 6.1.3 and the likelihood-ratio statistic from the EH-program, version 1.12 (Terwilliger and Ott, 1994). A gene frequency of 0.01, phenocopy frequency of 0.001, and a dominant disease transmission with 70% penetrance were assumed as specified parameters in the case-control sampling option of the EH-program. For stratified data with markers D13S292 and D13S787, the Bonferroni correction was applied by multiplying the P values with the number of alleles examined. Estimated haplotype frequencies were obtained from the EH-program (Terwilliger and Ott, 1994).

Study VI

For each of the 366 polymorphic markers in the screening, a contingency table was constructed for the observed counts of each allele in the cases and the controls, and Fisher's exact test (SPSS 9.0 software) was used in an initial search for association with AD. Markers showing some evidence of association ($P < 0.05$) were investigated more thoroughly. First, to test for overall association with AD, Pearson's chi-squared tests were performed. The significance of this measure of association was estimated by simulation, comparing the observed chi-squared statistic with 1000 replicates simulated under the null hypothesis of no association between marker and disease, by randomly permuting genotypes amongst cases and controls (Splus version 3.4). Concerns about the non-independence of counts and the small numbers in many cells of the table meant that comparing the test statistic with the asymptotic chi-squared distribution would be inappropriate, and so the significance was estimated by simulation. For each of 1000 simulations, 47 of the 98 individuals were randomly sampled and assigned to the 'Cases' group. A chi-squared statistic was computed for each simulation, and the significance was taken as the proportion of the chi-squared statistics which were greater than or equal to that recorded for the true data.

Secondly, to test for the association between a specific allele and the disease, each allele was in turn compared to the pooled group of other alleles. The case and control frequencies of the allele giving the highest chi-squared statistic (χ^2_{\max}) with Yates' correction were reported, along with odds ratios and asymptotic 95% confidence intervals. The significance of the linkage disequilibrium between this allele and the disease was again estimated by simulation, maximising the chi-squared statistic over all alleles in each simulation. 1000 simulated datasets were produced by randomly sampling cases, as for the overall association test. For each simulation, the chi-squared statistic was computed for the comparison of each allele with the pooled group of other alleles, and the maximum statistic was recorded. It is these simulated maximum chi-squared statistics, which were compared to χ^2_{\max} to obtain the simulated significance level. Maximising the chi-squared statistic over every allele for every simulation is necessary because the associated allele was not chosen a priori. The simulated significance hence takes into account the multiple tests performed at every marker. Thirdly, for each of these markers a flanking marker was identified, located within 3 cM of the initial marker. All flanking markers were similarly tested for overall and single allele associations.

Two-marker haplotypes were constructed for selected pairs of close markers, and the haplotype frequencies in cases and controls were calculated using the EH program version 1.12 (Terwillinger and Ott, 1994). For each pair, a likelihood ratio test for LD between the haplotype and the disease was performed, with the significance again estimated by the simulation of 1000 replicates. The haplotypes showing the most significant differences in frequency between cases and controls were identified, ranked on the basis of their chi-squared statistics.

Differences in the strength of the association between males and females, between those with or without an ApoE $\epsilon 4$ allele, and between those with familial and sporadic disease were tested using a case-only analysis. This provided an efficient test of the interactions. Formally, the binomial variable defining the sub-group was regressed on the marker genotypes of each case, using logistic regression (Splus version 3.4). The deviance of the model containing parameters for all alleles was compared with the deviance under the null model to obtain an asymptotic P-value.

All the LD markers were found to be in Hardy-Weinberg equilibrium in both AD and control groups except D2S405 and D3S2436. Genotype distribution of these markers displayed significant deviation from equilibrium in the D2S405 AD group ($P = 0.003$) and in the D3S2436 control group ($P = 0.017$). Reanalysis of markers D2S405 and D3S2436 did not reveal genotyping errors.

5. RESULTS

5.1. Molecular genetic analysis of the early onset AD family (Study I)

The fact that the AD family studied here showed clear autosomal dominant transmission accompanied with low disease onset ages (mean onset age 43 ± 2 years), lead us to focus our original molecular genetic studies on the PSEN-1 gene, which has been frequently found to be mutated in the familial form of early onset AD.

Sequencing of the coding region of the PSEN-1 gene revealed only a substitution E318G in exon 9 in two affected members of the early onset AD family and in eight healthy individuals (Figure 4). The segregation analysis of E318G in the family revealed genetic inconsistency in some individuals in which cases the substitution did not follow the normal Mendelian inheritance pattern. However, extended haplotype analysis conducted with microsatellite markers D14S77 and D14S1025 flanking 5' and 3' of the PSEN-1 gene, respectively, and with biallelic marker located at the intron 8, revealed a normal segregation pattern suggesting a heterozygote genomic deletion around the exon 9 starting downstream from the intron 8 polymorphism site (Figures 4 and 5). All of the AD patients possessed the same haplotype 5-2-del-2 for D14S77, intron 8 polymorphism, E318G, and D14S1025, respectively.

RT-PCR analysis using the lymphocyte RNA sample extracted from the subject with the disease-associated haplotype with exons 8 and 10 specific primers, revealed a truncated fragment (261 bp) in addition to a normal fragment (348 bp). Sequencing analysis of the truncated fragment showed an inframe deletion of codons 290-319 corresponding to exon 9 defining the locations of the genomic deletion break-point regions in the introns 8 and 9. Moreover, RT-PCR analysis with exons 7 and 12 specific primers showed a truncated fragment lacking the alternatively spliced exon 8 and also exon 9.

Long PCR amplifications with the different introns 8 and 9 specific primers were conducted to define the deletion break-point regions in the genomic level. Using the primers IN8F4 and IN9F5, which are located approximately 2.7 and 3.3-kb from exon 9 (Figure 5), respectively, produced a novel 1.6 kb PCR fragment, which was seen only in the affected member of the family. Multiplex PCR primers (DE8F1, DE8R2 and DE9R1) were designed on the basis of the sequence data and the screening of the affected members of the family with the disease-associated haplotype 5-2-del-2 revealed a heterozygous 290 bp fragment. Sequence analysis of the 290 bp fragment defined the genomic deletion to be 4555 bp large, consisting of a 1.6-kb distal part of intron 8, exon 9 and a 2.9-kb proximal part of intron 9 (Figures 5 and 6). Deletion screening analysis of 102 AD patients and 51 control subjects from Eastern Finland did not reveal any additional cases with the PSEN-1 exon 9 deletion.

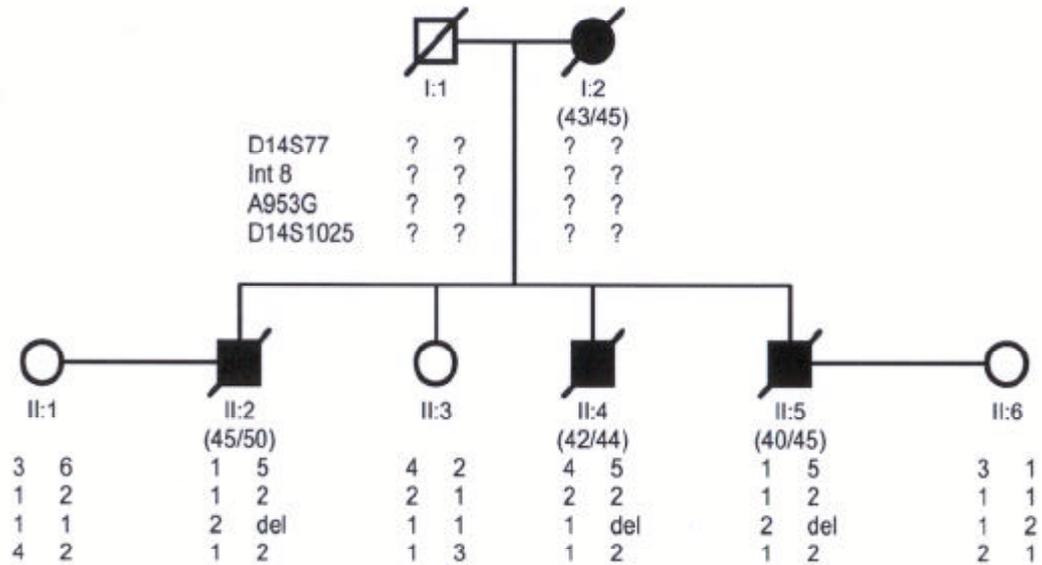


Figure 4. Haplotype analysis with markers flanking or within the PSEN-1 gene on chromosome 14. Affected individuals are denoted by a blackened symbol and the ages at onset and at death are indicated in the parenthesis, respectively. Haplotype 5-2-del-2 is shared in affected family members (II:2 = probable AD, II:4 = definite AD and II:5 = probable AD). Int 8 = intron 8 polymorphism, A953G = A (1) to G (2), a substitution leading to the amino acid change from glutamic acid to glycine at codon 318 (E318G). Generation III is not shown in the figure due to ethical reasons.

5.1.1. Repeat sequence analysis of the introns 8 and 9 of the presenilin-1 gene

A search for *Alu* consensus sequences and other repeat sequences from 5' breakpoint region showed that intron 8 shared extensive homology with the left *Alu* monomer (FLAM C) sequence, which also included a 26 bp *Alu* core sequence (Figure 6, Deininger et al., 1981). This highly conserved core sequence, which is suggested to promote recombinations (Rüdiger et al., 1995), was located in close proximity of the tetranucleotide sequence TGGG shared by both recombining segments. At the 3' breakpoint region (intron 9), a segment with a minor homology to *Alu* core sequence was found (Figure 6). Comparison of the deletion breakpoint regions of introns 8 and 9, however, did not reveal significant homology between sequences, which on the other hand would rule out the possibility of homologous pairing as an initial cause of the deletion. Other parts of intron 8 included numerous *Alu* consensus sequences composed of two homologous portions arranged in a head to tail dimer whereas no such sequences were found from intron 9 (Figure 5).

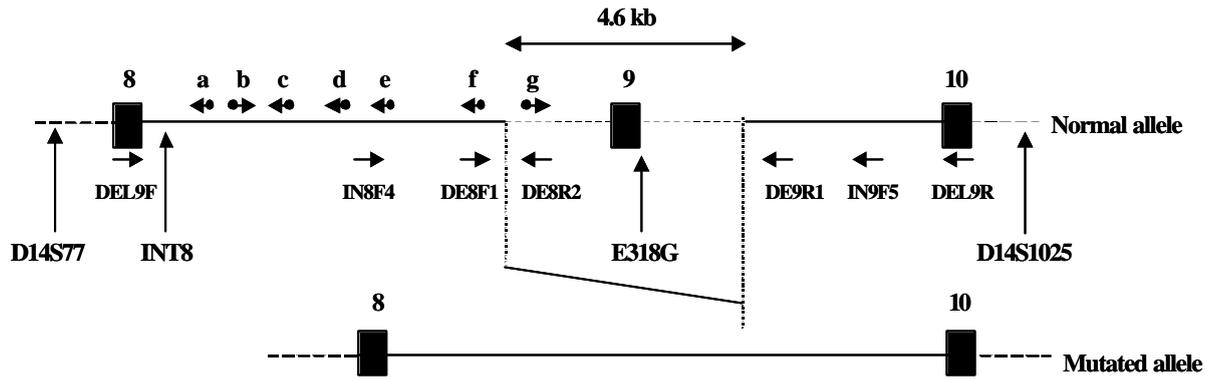


Figure 5. Schematic presentation of the normal and mutated alleles of PSEN-1 gene. The location and orientation of PCR primers (arrows) and human *Alu* consensus sequences (modified arrows) are indicated in the introns 8 and 9. The relative locations of D14S77, D14S1025, intron 8 polymorphism (INT8), and E318G substitution are indicated. *Alu* subfamily sequences are composed of two homologous portions arranged in a head to tail dimer of approximately 130 bp.

5.1.2. Neuropathology

Histopathological examination of the affected member of this family revealed characteristic lesions of AD in the gray matter. Silver stain demonstrated numerous neuritic plaques, diffuse plaques as well as senile plaques with central core formation. Furthermore, numerous neurofibrillary tangles (NFT) and neuropil threads (NT) were noted. No cotton wool plaques, i.e. rounded dense plaques without neuritic lesions, which are commonly seen in the variant AD, were observed. Alzheimer's degenerative changes were sufficient for the diagnosis of definite AD according to CERAD (Mirra et al., 1991).

Thioflavin-S staining visualized several amyloid cores and cerebral amyloid angiopathy (CAA), both in the parenchyme and in the leptomeninges. Beta-amyloid aggregates were visualized with immunohistochemical staining in the vessel walls of the neuropil and leptomeninges (CAA) and in the neuropil as plaques. These plaques were quite numerous, round in shape but varied in size. Numerous plaques and CAA were labelled with antibodies directed to β A4, β A42, 6E10 and 4G8 whereas only a few were labelled with antibodies against the short β A40. Some amyloid aggregates were also detected in the white matter. Numerous NFTs, NTs and neurites in neuritic plaques were visualized with phosphorylated PHF-Tau antibody. Furthermore, positive NTs were also seen in the white matter. Intraneuronal inclusions labelled with synuclein-1 or with α -synuclein antibodies could not be detected, even though the latter antibody labelled some of the plaques. Prion protein staining did not reveal the changes which are seen in prion diseases.

A quite intense inflammatory reaction was noted in the brain tissue seen as numerous activated microglial cells (AM) and reactive astrocytes (RA). AMs were primarily located centrally in the plaques, whereas the RAs were seen more diffusely within the gray matter. Both cell types were also identified within the white matter. Moderate complement factor labelling was observed in association to the plaques with antibodies directed to C3d factor,

weak staining with antibodies directed to C3c and C1q factors, whereas no staining was detected with antibodies directed to C5 complement factor.

5.2. Analysis of the presenilin-1 gene E318G substitution (Study II)

Sixteen unrelated familial AD patients and 64 sporadic neuropathologically confirmed definite AD patients as well as 270 clinically and neuropathologically verified control subjects were screened for the E318G substitution using the PCR-RFLP method. The E318G variant allele frequency was 25%, 11% and 4% in familial, sporadic and control cases, respectively, indicating a statistically increased allele frequency for E318G both in familial (Fisher's exact test, $P = 0.005$; OR 7.6, 95% CI 2.2-25.7) and in sporadic (Fisher's exact test, $P = 0.03$; OR 3.1 95% CI 1.1-8.2) AD groups.

Sequence analysis of the open reading frame exons of the PSEN-1 gene did not reveal any additional changes in four familial AD patients carrying the E318G substitution. Analyses with microsatellite marker D14S77 flanking the PSEN-1 gene and with a biallelic marker located at the 5' end of intron 8, showed that all of the AD patients and controls carrying the E318G substitution shared the same alleles, 203 bp and A for these markers, respectively. Corresponding allele frequencies in Eastern Finnish population were found to be 5% and 48% for markers D14S77 and intron 8 polymorphism, respectively.

Intron 8	GCATAAAGAA	AGAAAACCTA	ATAAATCGCT	GGTGGCTTCA	TTTCCAAAAG
Deletion	GCATAAAGAA	AGAAAACCTA	ATAAATCGCT	GGTGGCTTCA	TTTCCAAAAG
Intron 9	CAGCTCACAG	AGCATGCTGC	CCCAGCTGCA	CCTCCCCTGC	TGCAGCTGCC
Intron 8	GCTATGGAAA	TCCATAAACA	<u>GGctgggTGC</u>	AGTGGCTCAT	GCCTGTAATC
Deletion	GCTATGGAAA	TCCATAAACA	<u>GGctgggCCG</u>	CTGCTGCCAT	CAAGACCACT
Intron 9	<u>ATCTTTGCAG</u>	<u>CAGCCACTCC</u>	AGAtgggCCG	CTGCTGCCAT	CAAGACCACT
Intron 8	<u>CTAGCACTTT</u>	GGGAGGCTGA	GGCGGGAGGA	TTGCTTGAGC	CCAGGAGTTT
Deletion	<u>AGTAAACCTG</u>	ATAGAAAAGT	AAGAAATATA	CTGGAAAGAA	TTGCCACAAG
Intron 9	<u>AGTAAACCTG</u>	ATAGAAAAGT	AAGAAATATA	CTGGAAAGAA	TTGCCACAAG
Intron 8	GAGTCTGGCC	TGGGCAACGT	AGTGAGACCC	TGTTTTTAGA	AAAAATGAAG
Deletion	<u>CCTGGCTCTT</u>	GTGTTAACCT	GTGTTAAGCT	AAAGAAAATC	AAATGATTGT
Intron 9	<u>CCTGGCTCTT</u>	GTGTTAACCT	GTGTTAAGCT	AAAGAAAATC	AAATGATTGT
Intron 8	<u>ATAAAAAACT</u>	AATCCATAAA	CCTCATACTT	GGTGGTTGCC	CTTCAGTTTT
Deletion	<u>CTGTGAGCAT</u>	GTAGGTATAT	ATGTATGTGA	GTGAGTGATA	CAACAGAATT
Intron 9	<u>CTGTGAGCAT</u>	GTAGGTATAT	ATGTATGTGA	GTGAGTGATA	CAACAGAATT

Figure 6. Genomic sequences of PSEN-1 involved in the recombination process. Recombinogenic 26 bp *Alu* core sequence (double line) and a sequence displaying homology with FLAM C repeat sequence (underlined) located in intron 8 are shown. The presumed homology segment for *Alu* core sequence is indicated in intron 9 (dotted line). The tetranucleotide sequence TGGG (lower case letters) is shared by both recombining fragments.

5.3. Analysis of the butyrylcholinesterase K variant gene polymorphism (Study III)

Allele frequency of BChE K variant gene was examined in an unbiased group of AD patients and control subjects ≥ 65 years of age. The allele frequency of BChE K variant was 0.14 and

0.15 in AD and control groups consisting of 40 late onset AD patients and 51 age-matched controls from Eastern Finland (χ^2 , $P = 0.85$; OR 0.92, 95% CI 0.40-2.1).

In order to study the interaction between BChE K variant and ApoE $\epsilon 4$ alleles, 59 AD patients and control subjects carrying the ApoE $\epsilon 4$ allele were randomly selected for further studies. Genotype distributions of BChE K variant in whole AD group (including all of the AD patients ≥ 65 years of age) and in the AD patient group ≥ 75 years of age did not reveal any statistically significant differences when compared to corresponding control groups. However, comparison of allele frequencies of BChE K variant revealed statistically significant difference between AD patient (12%) and control (22%) groups ≥ 65 years of age (χ^2 , $P = 0.037$; OR 0.48, 95% CI 0.23-0.97), but not in AD patient (18%) and control (22%) groups ≥ 75 years of age (χ^2 , $P = 0.65$; OR 0.79, 95% CI 0.28-2.18). To determine whether the difference observed in whole AD group was due to the presence of patients who were under 75 years of age, genotype and allele frequencies of BChE K variant were also studied independently in this subgroup. These data revealed a statistically significant difference in the BChE K genotype and allele frequencies between AD patient (allele frequency = 8%) and control (allele frequency = 22%) groups (for genotype data χ^2 , $P = 0.02$ and for allele data χ^2 , $P = 0.02$; OR 0.31, 95% CI 0.11-0.86). Considering only the carrier status of BChE K and ApoE $\epsilon 4$ alleles, the proportion of subjects carrying both alleles in the AD patient and control groups under 75 years of age was 5/37 (14%) and 14/34 (41%), respectively (χ^2 , $P = 0.009$; OR 0.22, 95% CI 0.07-0.71).

5.4. ApoE -491A/T promoter polymorphism (Study IV)

Genotype and allele frequencies of ApoE $\epsilon 2/3/4$ and ApoE -491A/T promoter polymorphisms were assessed from 106 late onset AD patients and 123 age-matched control subjects. The distribution of $\epsilon 2/3/4$ alleles of ApoE in the AD and control groups was 0.01/0.53/0.46 and 0.02/0.80/0.18, respectively (χ^2 , $P < 0.001$) indicating that the $\epsilon 4$ allele was significantly associated with AD (χ^2 , $P < 0.001$; OR 5.9, 95% CI 3.3-10.4). On the basis of variance analysis, AD patients with two $\epsilon 4$ alleles had earlier onset age (66.8 ± 3.3 years) than AD patients with one (73.2 ± 5.8 years) or no (74.6 ± 3.9 years) $\epsilon 4$ alleles (Duncan, $P < 0.05$).

Genotype and allele distributions of the -491A/T promoter polymorphism did not reveal difference either between the overall series or between AD patient and control groups, which were stratified according to ApoE $\epsilon 4$ status (ApoE 23/33 and ApoE 24/34/44). In addition, using the pooled data of the -491TT/TA genotypes as reference, no statistically significant increase in -491AA genotype frequency was observed either in overall (χ^2 , $P = 0.06$; OR 1.9, 95% CI 1.0-3.9), ApoE 23/33 (χ^2 , $P = 0.09$; OR 2.9, 95% CI 0.8-10.7) or ApoE 24/34/44 (χ^2 , $P = 0.34$; OR 1.6, 95% CI 0.6-4.0) groups when compared to the corresponding control groups.

In order to study if there was linkage disequilibrium between ApoE and -491A/T alleles, all the AD patients and control subjects were pooled into one group and the genotype distributions between ApoE and -491A/T polymorphism were determined. This analysis indicated a linkage disequilibrium between ApoE and -491A/T alleles (χ^2 , $P < 0.001$). Linkage disequilibrium between ApoE and -491A/T polymorphism alleles was further assessed using haplotype estimation analysis. According to the estimation analysis, the haplotype distribution differed significantly between AD and controls groups (χ^2 , $P < 0.001$),

and the haplotype ApoE $\epsilon 4/-491A$ conferred the highest risk whereas haplotypes ApoE $\epsilon 2$ and $\epsilon 3/-491T$ encompassed the lowest risk in AD group.

5.5. Genome-wide linkage disequilibrium mapping (Studies V-VI)

In order to find novel gene loci associated with AD, genome-wide linkage disequilibrium (LD) mapping was performed using 47 late onset AD patients and 51 age-matched control subjects. These AD patients and controls were collected from a restricted area of eight communities in the province of Northern Savo in Eastern Finland, where the population has descended from a small group of original founders who migrated to the region in the late 16th and early 17th century.

The distribution of ApoE $\epsilon 2/3/4$ alleles was determined to be 0.02/0.62/0.36 and 0.02/0.83/0.15 in AD and control groups, respectively (χ^2 , $P = 0.002$) indicating that ApoE $\epsilon 4$ allele was significantly associated with AD (χ^2 , $P = 0.001$; OR 3.3, 95% CI 1.6-6.6).

Initial genome-wide LD screening of autosomes and X chromosome with 366 polymorphic microsatellite markers revealed a total number of 22 chromosomal loci associated with AD with Fischer's exact test P-values less than 0.05 (data not shown and Table 4). In the overall association test, six of these chromosomal loci displayed simulated P values < 0.01 . Comparison of single allele frequencies of the microsatellite markers in AD and control groups indicated the presence of risk alleles displaying suggestive association with AD (odds ratio > 1) as well as protective alleles (odds ratio < 1) in which case the particular allele frequency was significantly lower in the AD group. Eleven of these markers showed a statistically significant single allele association with AD (simulated P-value < 0.05 , Table 4).

Screening of the 22 LD regions with additional markers, located 0-3 cM distance from the initial loci, revealed eight chromosomal loci (1p36, 2p22, 3q28, 4p12, 10p13, 13q12, 18q12 and 19p13) that were associated with AD more than one microsatellite marker (Table 4). Although some of the flanking markers displayed only borderline significance in the overall and/or single allele tests, these markers were included to perform a haplotype estimation analysis. One of these LD regions located at the 13q12, pointed to stronger single allele associations of microsatellite markers D13S292 and D13S787 with AD in the female and familial groups than in the unstratified group. Combined female and familial data indicated overlapping effect for the 13q12 locus, which was accompanied with statistically significant single allele association with AD even after the adjustment for multiple comparisons (data not shown). In addition, logistic regression analysis among cases revealed that the allele frequencies of markers D1S552 (female/male), D4S3251 (female/male), D18S877 (ApoE4⁺/ApoE4⁻), and D19S1034 (familial/non-familial) varied significantly between subgroups. The most significant association was between D19S1034 and familial/non-familial disease status ($P = 0.004$).

Although the marker phases of individual AD patients and controls were not known, haplotype estimation analyses were conducted with the AD-associated marker pairs located in the different chromosomal loci using the EH program (Table 5). On the basis of the estimation analyses, haplotype distribution of markers D1S552-D1S2644, D3S1602-D3S2436, and D19S549-D19S1034 indicated statistically significant differences between AD and control chromosomes. Comparison of individual haplotype frequencies revealed over-representation of haplotype 7-1 of markers D3S1602-D3S2436 in the AD chromosomes, whereas haplotypes 2-5 of D4S1627-D4S3251 and 6-6 of D18S66-D18S877 were more

frequent in the control chromosomes. In the case of 13q12 locus, female/familial stratified genotype data revealed that the haplotype 5-1 of the markers D13S292 and D13S787 occurred in 13% of AD chromosomes, but only in 1.5% of control chromosomes.

Table 4. Microsatellite markers found to be in linkage disequilibrium (LD) in different chromosomes and odds ratios (OR) calculated for associated alleles.

Marker ^a	Locus	No. of Alleles	Overall LD P-value	Allele ^b	Allele frequency ^c		OR	95% CI	P-value ^d
					AD	Control			
D1S552	1p36.12	5	0.006	5	0.09	0.00			0.018
D1S2644*	1p36.12	10	0.072	2	0.15	0.06	2.80	1.03-7.62	0.25
D1S1679	1q24.2	10	0.038	2	0.16	0.03	6.27	1.75-22.41	0.015
D2S171*	2p22.2	13	0.021	4	0.12	0.03	4.37	1.18-16.20	0.183
D2S405	2p22.1	5	0.009	5	0.13	0.03	4.83	1.32-17.70	0.045
D3S1602*	3q28	13	0.198	7	0.32	0.14	2.95	1.45-6.00	0.007
D3S2436	3q28	5	0.021	1	0.22	0.08	3.38	1.41-8.07	0.013
D3S2418	3q29	5	0.017	1	0.57	0.40	2.01	1.14-3.55	0.047
D4S1627	4p12	7	0.004	2	0.19	0.36	0.42	0.22-0.80	0.039
D4S3251*	4p12	6	0.132	5	0.16	0.28	0.48	0.24-0.96	0.106
D5S807	5p15.2	9	0.001	2	0.63	0.49	1.75	0.99-3.10	0.155
D6S1017	6p21.1	11	0.003	5	0.00	0.08	0.00	0.00-0.00	0.039
D6S1960	6p12.1	8	0.039	5	0.57	0.36	2.37	1.34-4.21	0.012
GAAT1A4	8q22.3	5	0.028	4	0.25	0.39	0.50	0.27-0.93	0.080
D10S1664*	10p13	11	0.020	1	0.02	0.15	0.13	0.03-0.57	0.007
D10S674	10p13	9	0.042	5	0.01	0.09	0.11	0.01-0.90	0.133
D13S292*	13q12	5	0.001	2	0.14	0.04	3.93	1.23-12.53	0.044
D13S787	13q12	5	0.034	5	0.00	0.05	0.00	0.00-0.00	0.235
D14S77	14q24.3	26	0.048	5	0.07	0.00			0.062
D15S659	15q21.1	10	0.036	7	0.21	0.11	2.24	1.01-4.96	0.255
D16S403	16p12.1	14	0.012	5	0.18	0.07	3.00	1.18-7.60	0.156
D17S1293	17q11.2	10	0.025	3	0.27	0.12	2.72	1.28-5.79	0.039
D18S59	18p11.32	11	0.009	3	0.00	0.056	0.00	0.00-0.00	0.201
D18S976	18p11.31	9	0.032	6	0.23	0.13	2.10	0.99-4.44	0.172
D18S66*	18q12.1	10	0.025	6	0.11	0.25	0.35	0.16-0.77	0.032
D18S877	18q12.1	6	0.038	6	0.27	0.43	0.48	0.26-0.87	0.052
D19S549*	19p13.3	5	0.060	1	0.07	0.02	4.02	0.81-19.88	0.300
D19S1034	19p13.3	6	0.057	6	0.07	0.22	0.29	0.12-0.72	0.013
D19S433	19q13.11	9	0.062	1	0.00	0.09	0.00	0.00-0.00	0.021
D20S604	20p11.23	7	0.036	4	0.26	0.14	2.16	1.04-4.47	0.161

^a Microsatellite markers used in the second screening are indicated as asterisks.

^b Marker allele displaying the strongest association in the single allele test.

^c Frequencies of the allele in AD (n = 94) and control (n = 102) groups giving the strongest single allele association.

^d Simulated p-value for associated allele obtained from single allele test.

Table 5. Estimated haplotype frequencies of the AD (94) and control (102) chromosomes.

Markers and haplotype ^a	Haplotype frequency		Markers and haplotype ^a	Haplotype frequency	
	AD	Control		AD	Control
D1S552-D1S2644 P = 0.006 (1.6 cM)			D2S171-D2S405 P = 0.126 (2.5 cM)		
3 - 2	0.134	0.013	8 - 3	0.036	0.155
3 - 8	0.175	0.049	4 - 3	0.047	0.000
4 - 6	0.105	0.012	12 - 3	0.021	0.079
5 - 9	0.068	0.000	6 - 3	0.113	0.046
3 - 6	0.000	0.075	8 - 4	0.064	0.017
D3S1602-D3S2436 P = 0.162 (2.1 cM)			D4S1627-D4S3251 P = 0.02 (0 cM)		
8-2	0.067	0.195	2 - 5	0.000	0.135
7-2	0.210	0.076	6 - 5	0.048	0.000
7-1	0.100	0.021	5 - 6	0.087	0.022
9-2	0.000	0.055	7 - 6	0.034	0.000
10-2	0.053	0.000	4 - 3	0.011	0.057
D10S1664-D10S674 P = 0.312 (2.0 cM)			D18S66-D18S877 P = 0.094 (1.5 cM)		
1 - 3	0.000	0.062	6 - 6	0.049	0.186
6 - 4	0.125	0.041	5 - 5	0.288	0.127
6 - 10	0.000	0.051	3 - 5	0.000	0.064
6 - 3	0.161	0.076	5 - 8	0.000	0.038
9 - 3	0.000	0.045	7 - 6	0.000	0.035
D19S549-D19S1034 P = 0.021 (0.7 cM)					
1 - 5	0.064	0.000			
4 - 6	0.000	0.057			
5 - 4	0.000	0.047			
3 - 6	0.053	0.119			
3 - 3	0.032	0.000			

^a The estimated distances between markers in centiMorgans (cM) are indicated in parentheses. The five haplotypes presented are chosen as those, which give the highest chi-squared statistics.

6. DISCUSSION

6.1. Presenilin-1 gene analyses

6.1.1. Identification of a novel 4.6-kb genomic deletion in the presenilin-1 gene

Molecular genetic analysis of the Finnish early onset AD family members revealed a novel 4.6-kb genomic deletion in the PSEN-1 gene, which leads to the inframe exclusion of exon 9 ($\Delta 9$) from the mRNA transcript. At the mRNA level, this mutation results in a similar alteration as described for the variant AD (Crook et al., 1998) and the $\Delta 9$ splice mutations (Perez-Tur et al., 1995, Kwok et al., 1997, Sato et al., 1998). Recently, Prihar et al. (1999) defined the $\Delta 9$ mutation found in the Finnish variant AD pedigree to be exactly the same at the genomic level as the mutation described in the present early onset AD family. Although the genetic alteration was the same in both studies, the phenotypic features of the AD patients were not comparable with each other. In the present AD family, the neuropathological and clinical features of patients resembled the typical AD rather than variant AD. In this context, however, it should be considered that the brain biopsy from the restricted cortical region was taken only from one AD patient, which may limit the neuropathological extrapolation to the whole brain scale. Nevertheless, numerous neuritic plaques, neurofibrillary tangles and reactive astrocytes and microglial cells in association with the plaques were seen whereas the common clinical manifestations of the AD patients were the impairment of memory and the rapid progression of the disease after its onset. More importantly, there were no indications of spastic paraparesis or unusual eosinophilic ‘cotton wool’ plaques, which are the hallmarks of the variant form of AD (Crook et al., 1998, Verkkoniemi et al., 1999). Therefore, differences in the phenotypic features between $\Delta 9$ mutation families suggest that there may exist modifying genetic factors in addition to the main causative agent, which could contribute to the underlying disease process and the phenotypic outcome of AD in a context dependent manner.

Consistent with the previous idea, it has been suggested that the age of onset of disease induced by the PSEN-1 mutation could be influenced by other genetic factors either in a *cis*- or *trans*-acting way (Mehta et al., 1998). One potential candidate in this context could be the substitution of E318G in the PSEN-1 gene, which was found in two affected members of the family. Previous studies have suggested that the E318G substitution itself is a non-pathogenic alteration in AD as it has been found also from the healthy control population (Mattila et al., 1998, Aldudo et al., 1998, Dermaut et al., 1999). Although the E318G substitution does not seem to affect onset ages in this particular family and the mean onset age correlates well with that found with the $\Delta 9$ splice mutation family (Perez-Tur et al., 1995), our study using Eastern Finnish AD and control population suggests that the substitution could be a risk factor in AD as the allele frequency of the variant is significantly increased both in the familial and sporadic AD patient groups (see more details in the chapter 6.1.2). It is therefore possible that another change located in the promoter or regulatory region of the PSEN-1 gene, which is in linkage disequilibrium with the E318G substitution, could influence the PSEN-1 gene functions such as the expression status, which in turn could modify the phenotypic outcome. Interestingly, two of the affected members as well as the genetically unrelated individual who carried the E318G substitution in the family shared the same haplotype 1-1-2-1 for markers flanking the PSEN-1 gene over several centiMorgans region (Figure 4) indicating that there may exist a conserved founder haplotype, which segregates with the variant allele.

The well-conserved region of the 26 bp *Alu* core sequence has been considered to be a hot spot of recombination due to the fact that it is frequently found at or in the vicinity of those sites involved in homologous or non-homologous recombination (Rüdiger et al., 1995). Accordingly in this present study, a complete 26 bp *Alu* core sequence at intron 8 and a segment displaying homology also with the core sequence at intron 9 were found in close proximity to the tetranucleotide sequence TGGG shared by both recombining fragments. Subsequent comparison of the 5' and 3' sequences involved in the recombination process, however, did not reveal any major homology between these fragments, supporting the concept of *Alu* core-stimulated non-homologous recombination rather than *Alu* core-mediated homologous pairing of the fragments.

6.1.2. Increased frequency of the presenilin-1 gene E318G substitution in AD

The role of the E318G substitution (a substitution leading to the amino acid change from glutamic acid to glycine the codon 318) in the PSEN-1 gene has been difficult to resolve when compared to other missense mutations found in the coding region of the gene. With the exception of the E318G substitution, missense mutations found throughout the PSEN-1 coding region show a complete penetrance and co-segregation with the disease in an autosomal dominant manner in all of the AD families studied, confirming the causative nature of these mutations (Cruts and Van Broeckhoven, 1998). Although the E318G substitution has been frequently found in the early onset AD families, the onset ages have varied significantly between the substitution carriers and the co-segregation of the substitution with AD has not been demonstrated. In addition, *in vitro* data have revealed that the secretion of amyloidogenic A β 42 peptide is not increased in human embryonic kidney cells stably expressing the E318G PSEN-1 when compared to the corresponding wild type PSEN-1 cell line (Dermaut et al., 1999).

Recent genetic studies have suggested that the substitution could be a rare polymorphism instead of a pathogenic mutation since it is found also in the healthy control population (Mattila et al., 1998, Aldudo et al., 1998, Dermaut et al., 1999). Consistent with these studies, our results using Eastern Finnish AD patients and control subjects indicate that the E318G substitution is not a causative mutation in AD. In contrast to previous studies, however, screening analysis of the substitution revealed a statistically significant increase in the frequency of the substitution both in the familial and sporadic AD patient groups when compared to the age-matched control group suggesting that substitution is associated with an increased risk of developing AD. The increased frequency of the E318G substitution in the Eastern Finnish AD population could reflect the situation in which the substitution is in linkage disequilibrium with the change located in some other part of PSEN-1 gene. The potential location of the change affecting the PSEN-1 gene function could be considered to be restricted to the promoter or other regulatory regions of the gene due to the fact that the sequence analyses of the coding region have not revealed any additional changes in subjects carrying the substitution. In this respect, it is crucial to assess novel polymorphisms found in the 5' regulatory region of the PSEN-1 gene in AD patients carrying the E318G substitution since Theuns et al. (2000) have suggested that two of these variations increase the risk for early onset AD by altering the expression levels of the PSEN-1 protein.

Analyses with the microsatellite marker D14S77 and with the intron 8 biallelic marker showed that all the E318G substitution carriers (patients and controls) shared the same alleles (203 bp and A, respectively). These alleles were also the same in the case of the Δ 9 mutation family members carrying the substitution-associated haplotype 1-1-2-1 (Figure 4)

emphasizing that E318G substitution carriers may share a common founder. In Finland there are regional population bottlenecks which have created clusters of several Finnish heritage diseases and the carrier frequencies of these disease mutations reveal a distinct geographic location (Peltonen 1999, Pastinen et al., 1999). This may explain why the frequency of the E318G substitution is different in Finland between Eastern and South-Western AD populations (Mattila et al., 1998). Although these above-mentioned results and hypotheses concerning the E318G substitution are compelling, more data are needed before one can draw any conclusions on the importance of the substitution in AD.

6.2. Susceptibility gene analyses

6.2.1. Protective effect for butyrylcholinesterase K variant allele in AD

BChE K variant allele was initially considered to act in synergy with the ApoE ϵ 4 allele in late onset AD (Lehmann et al., 1997). This effect was more pronounced among AD patient groups, in which the disease onset age was over 75 years, giving an odds ratio of 12.8 for those patients carrying both the BChE K variant and ApoE ϵ 4 alleles. This genetic finding fits well into the context of AD as the involvement of BChE in the underlying disease process is strongly supported by the fact that it is associated with neurofibrillary tangles and amyloid plaques as well as its participation in the transformation process of the A β from an initially benign form to the malignant β -plated form (Gómez-Ramos et al., 1994, Mesulam et al., 1994, Guillozet et al., 1997). Since the initial findings of Lehmann et al. (1997), however, numerous genetic studies have revealed contradictory results, either for or against the interaction between BChE K and ApoE ϵ 4 alleles in AD in various ethnic populations.

Despite the fact that the evaluation of the BChE K variant allele frequency did not reveal any difference between Finnish late onset AD patients and control subjects in the unbiased populations, additional AD patients and control subjects were selected for further study according to their ApoE ϵ 4 status. The results obtained with ApoE ϵ 4 carriers, however, did not show synergy between the BChE K variant and the ApoE ϵ 4 alleles even after stratification according to the age of onset. In contrast, the BChE K variant allele frequency was significantly reduced in AD patients group under 75 years of age and carrying the ApoE ϵ 4 allele when compared to corresponding age-matched controls suggesting that the BChE K allele had a protective effect in this subgroup.

Whether these opposite results in different populations with respect to the BChE K variant in AD are due to linkage disequilibrium between the BChE K variant gene and some other susceptibility gene near the BChE locus, sampling strategy or the ethnic origin of samples remain to be determined. On the other hand, it has been shown that BChE participates together with acetylcholinesterase (AChE) in the hydrolysis of acetylcholine (ACh) (Atack et al., 1986) and that AD patients carrying the ApoE ϵ 4 risk allele suffer from a severe cholinergic deficit (Soininen et al., 1995a). The protective effect of the BChE K variant could then be considered to be due to the fact that the K variant has 30% less catalytic activity than wild-type BChE (Bartels et al., 1992), which in turn would lead to increased concentration of ACh in the brain. According to this idea, the BChE K variant with reduced enzymatic activity would therefore provide some protection for cholinergic neurotransmission in subjects carrying the ApoE ϵ 4 risk allele. Experimental data, however, do not support the above-mentioned concept as the activity of AChE is increased in cerebrospinal fluid of late onset AD patients carrying the ApoE ϵ 4 allele (Soininen et al., 1995b). Consequently, increased

enzymatic activity of AChE would then be expected to compensate for the reduced effect of BChE K variant in the hydrolysis of ACh.

6.2.2. Linkage disequilibrium between ApoE -491 A/T promoter and ApoE alleles

Sequence analyses of the ApoE regulatory region have revealed several novel polymorphisms, which have been proposed to be involved in differential expression of the ApoE mRNA (Artiga et al., 1998b, Bullido et al., 1998, Lambert et al., 1998b). One of these polymorphisms, -491A/T, has been shown to associate with increased risk for AD in its AA homozygosity state and to act independently of the ApoE ϵ 4 allele status (Bullido et al., 1998). Furthermore, according to the transient transfection studies in human hepatoma and astrocyte cell lines, the -491A allele was also associated with increased constitutive levels of ApoE promoter activity, more than the T allele, suggesting that the -491A/T polymorphism may modify the risk for AD by altering the level of ApoE protein expression.

The assessment of Apo E gene promoter -491A/T polymorphism and its dependence on the ApoE ϵ 4 allele in Finnish late onset AD patients failed to detect any statistically significant differences either in the whole material or in the ApoE ϵ 4 stratified subgroups when compared to age-matched controls. However, it should be mentioned that the failure to detect an independent effect of -491 A/T polymorphism in this present study may be related to the lack of statistical power due to the small sample sizes in different subgroups. Although the -491 A/T polymorphism did not confer an independent risk for AD in this study population, the ApoE allele distribution demonstrated the expected increase in the ApoE ϵ 4 allele frequency and earlier onset ages due to the gene dose effect in AD patients group. Consistent with previous studies (Town et al., 1998, Roks et al., 1998, Rebeck et al., 1999, Thome et al., 1999, Zurutuza et al., 2000), the -491 A/T polymorphism appeared to be in linkage disequilibrium with the ApoE coding region polymorphisms according to the significantly elevated proportion of different ApoE and the -491 AA genotypes. In addition, the haplotype ϵ 4/A conferred the highest risk in AD due to the increased frequency of this particular haplotype in the AD patient group (46%) when compared to the control group (17%). These haplotype frequencies were in turn the same as the independent ApoE ϵ 4 allele frequencies in AD and control groups. Collectively these data suggest that the effect of the -491 A/T polymorphism can be explained by the presence of the A allele, which is in linkage disequilibrium with the ApoE ϵ 4 allele.

6.3. Genome-wide linkage disequilibrium mapping of late onset AD

The initial genome-wide LD screening of Eastern Finnish late onset AD patients and controls revealed 22 chromosomal loci, which were found to be associated with AD with varying degrees of statistical significance ($P < 0.05$). To assess the extent of the LD in these regions in more detail, additional microsatellite markers were genotyped in the vicinity (0-3 cM) of the associated markers. Chromosomal loci in 1p36.12, 2p22.2, 3q28, 4p13, 10p13, 13q12, 18q12.1, and 19p13.3 indicated suggestive association with more than one microsatellite markers and the subsequent two-marker haplotypes estimation analyses revealed over- as well as under-representation of particular haplotypes in the group of AD patients. Interestingly, a few of the microsatellite markers demonstrated variation in genotype frequencies with ApoE allele, sex and familial/non-familial status, pointing to possible interactions of these different factors in the underlying disease process.

The ApoE $\epsilon 4$ allele frequency was significantly increased in the AD group when compared to the control group, verifying that the population structure in this geographically stratified AD and control material was not biased due to the initial sampling strategy. However, microsatellite marker D19S178, which is located approximately 2.5 cM from the ApoE gene locus and was the nearest marker included in the original screening set, did not reveal any overall LD or single allele association with AD either in the unstratified or in the ApoE $\epsilon 4$ stratified groups. Further screening with the marker D19S219, located approximately 1.0 cM distally from the ApoE locus, revealed an increased frequency of the 162 bp allele among the AD patient group (0.42) when compared to the control group (0.29). Although this increase was not statistically significant in the unstratified group ($P = 0.10$; OR 1.70, 95% CI 0.94-3.10), the frequency of this particular allele was found to be significantly greater in the AD group carrying the $\epsilon 4$ allele (0.50) than in the non-carrier AD group (0.29) (χ^2 , $P = 0.04$).

These results obtained with microsatellite markers around the ApoE locus and also with our -491 A/T ApoE promoter association study suggest that detecting LD in the vicinity of ApoE $\epsilon 4$ allele is not a straightforward task even in a genetically homogenous population. The fact that the LD can be detected at very small distances around the ApoE $\epsilon 4$ allele locus probably reflects the evolutionary ancient nature of this allele, originating before the divergence of present day populations 100 000 years ago (Gong et al., 1999). Accordingly, it is possible that the chromosomal regions (0-2.5 cM) found to be associated with AD in the present genome-wide scan would contain susceptibility gene alleles, which were introduced more recently into the population and thus permit the detection of LD between population-specific disease alleles and marker alleles over larger chromosomal regions than with the ApoE locus.

Consistent with previous mapping studies (Pericak-Vance et al., 1997, Zubenko et al., 1998, Garcia et al., 1999), our genome-wide screen revealed overlapping regions on chromosomes 6 and 10 with the markers D6S1017, D10S1664, and D10S674 (Table 1). The locus at which we found LD on chromosome 10 (D10S1664 and D10S674) is located approximately 1 cM from the marker D10S1423, which was found to be associated with AD in the studies of Zubenko et al. (1998) and Majores et al. (2000). Interestingly, we also found an association with the marker D14S77, which is located in the vicinity (< 0.5 cM) of the PSEN-1 gene. Thus, our observation that the PSEN-1 substitution E318G is a risk factor among Finnish familial and sporadic AD patients could in fact reflect a situation in which E318G and D14S77 are in LD with the same predisposing change affecting the PSEN-1 gene (see more details in the chapter 6.1.2). Also, a transmembrane glycoprotein called nicastrin, which was recently described to modulate presenilin-mediated notch/glp-1 signal transduction and APP processing maps to the D1S1595-14cM-D1S2844 region on chromosome 1 (Yu et al., 2000). This region contains the marker D1S1679, which showed significant evidence of association in our present genome-wide study.

Characterization of the known genes located within 1.5 Mb of the markers found to be associated with AD revealed 16 potential candidate genes with functions which have been suggested to be important in the pathogenesis of AD. These included genes that are involved in inflammation, neurotransmission, oxidative stress and cytoskeleton structures. In addition, over 20 genes, which are indirectly linked (e.g. via signal transduction pathways) with AD or other neurodegenerative disorders are located in close vicinity to 22 LD loci. In the case of the 13q12 locus, only two currently known genes expressed in the brain (H,K-ATPase α -subunit protein and $\alpha 2$ -tubulin) and two infant brain-derived expressed sequence tags (EST) are located in this particular region, making them attractive candidates for further sequencing and functional analyses.

Despite the fact that observations of possible AD-risk loci made in this study are partly consistent with previously described findings, it must be stated that the microsatellite markers found to be associated should be viewed with caution. In purely statistical terms, even if none of the 366 markers in the original screen were associated with AD, we would still expect around 18 to produce P-values below the conventional 5% threshold. Also, the multiple testing issues and the limited sample size are factors that clearly influence the credibility of our results as a whole. For example, small sample sizes may have affected the statistical power to detect the 13q12 locus effect on male and ApoE4⁺/ApoE4⁻ subgroups.

It has been suggested that detecting a region of markers displaying P-values nearing the significance for association could be a critical indicator in distinguishing true positives from a false positive in the presence of an actual disease gene (Terwilliger et al., 1997). In that sense, this study has identified eight regions of the genome meriting further study, and it is anticipated that candidate genes located in close proximity to these loci will provide the primary targets for future genetic and functional studies into this disease.

7. SUMMARY AND CONCLUSIONS

1) Molecular genetic analyses of the PSEN-1 gene in an early onset AD family identified a novel 4.6-kb genomic deletion in the PSEN-1 gene resulting in exclusion of exon 9 ($\Delta 9$) from the mRNA transcript. This mutation was clearly a causative mutation for AD. The underlying recombination mechanism in this $\Delta 9$ alteration was considered to involve an *Alu* core sequence-stimulated non-homologous genomic rearrangement. Mutation screening analysis did not reveal further cases with the $\Delta 9$ mutation, indicating that the frequency of this alteration is low among the Eastern Finnish AD population. Phenotypic features of the AD patients in the $\Delta 9$ family resembled those of the typical AD without indications of spastic paraparesis or 'cotton wool' plaques, which are seen in the variant form of AD. The expression of both normal and deleted PSEN-1 transcripts implies that pathogenic effects are not exerted through haploinsufficiency.

2) Although the E318G substitution in the PSEN-1 gene was found to be a non-causative mutation in AD, the increased frequency of the substitution both in the sporadic and familial AD patient groups suggests that the E318G substitution could be a risk factor in AD.

3) BChE K variant and ApoE $\epsilon 4$ alleles do not act in synergy in Finnish late onset AD patients. In fact, the BChE K variant allele frequency was significantly reduced in AD patient group under 75 years of age and carrying the ApoE $\epsilon 4$ allele suggesting a protective effect for BChE K variant allele in this subgroup.

4) The genotype and allele distributions of the ApoE gene promoter -491A/T polymorphism did not significantly differ between late onset AD patients and age-matched control subjects. In agreement with previous studies, -491 A/T and ApoE alleles were found to be in linkage disequilibrium, confirming that the ApoE $\epsilon 4$ status is still the strongest predictor of risk in Finnish late onset AD patients.

5) The initial genome-wide screening with 366 polymorphic microsatellite markers revealed 22 chromosomal loci associated with AD with P-values < 0.05 . Subsequent comparison of single allele frequencies of the microsatellite markers in the AD and control groups revealed the presence both of possible risk alleles (odds ratio >1) and of possible protective alleles (odds ratio <1). Screening of the 22 LD regions with additional microsatellite markers revealed eight chromosomal loci in 1p36.12, 2p22.2, 3q28, 4p13, 10p13, 13q12, 18q12.1 and 19p13.3 to be associated with AD in more than one microsatellite marker. A subsequent two-marker haplotype estimation analysis indicated over- as well as under-representation of particular haplotypes in this group of AD patients.

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