Genetic Variation of Transthyretin and ApoE Genes in Thai People

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ชื่อวิทยานิพนธ์	ความหลากหลายของยีน Transthyretin และ ApoE ในคนไทย
ผู้เขียน	สุภลักษณ์ ศรีนวล
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บทคัดย่อ

Amyloid β (Aβ) เป็นองค์ประกอบหลักของ senile plaques ในผู้ป่วยโรคอัล-ไซเมอร์ โดยพบว่า amyloid precursor protein (APP) ที่สร้างมากขึ้นซึ่งชักนำให้เกิด Aβ เพิ่มสูงขึ้นนั้นรวมทั้งการกลายพันธุ์ของยีนที่เกี่ยวข้องกับการสร้างโปรตีน amyloid ก็เป็นสาเหตุหลักของการเกิดก้อน amyloid ที่อวัยวะต่างๆโดยเฉพาะสมองด้วย ดังนั้นคนที่เป็นโรคพันธุกรรมบางโรคเช่น Down's syndrome (trisomy 21) ซึ่งมีโครโมโซมที่ 21 อยู่ 3 แท่งจึงมีความเสี่ยงสูงต่อการพัฒนาไปสู่การเกิดโรคอัลไซเมอร์ได้ ในบรรดาโปรตีนที่พบในพลาสมานั้น apolipoprotein E (ApoE) และ transthyretin (TTR) เป็นโปรตีนที่มีความสำคัญที่เกี่ยวข้องกับการเกิดก้อน amyloid โดยมีการยืนยันถึงความสัมพันธ์ระหว่างอัลลีลของยีน ApoE โดยเฉพาะ ApoE-ϵ4 กับโรคอัลไซเมอร์ พบว่าความสัมพันธ์นี้มีความแตกต่างในระหว่างกลุ่มเชื้อชาติ สำหรับประเทศไทยนั้นเคยปรากฏรายงานของลักษณะจีโนไทป์ของยีน ApoE เฉพาะในกลุ่มคนปกติและกลุ่มผู้สูงอายุเท่านั้น ส่วนความหลากหลายของยีน TTR ก็พบในทำนองเดียวกัน โดยพบว่า TTR เป็นองค์ประกอบหลักของการเกิดก้อน amyloid โดยเฉพาะอย่างยิ่ง ณ บริเวณปลายประสาทและนำไปสู่อาการในกลุ่ม familial amyloid polyneuropathy (FAP), familial amyloid cardiomyopathy (FAC) และ senile systemic amyloidosis ซึ่งมีความแตกต่างกันในระหว่างกลุ่มเชื้อชาติเช่นกัน โดยพบว่า TTR เกิดการกลายพันธุ์ได้มากกว่า 80 ตำแหน่ง ด้วยความถี่ของการเกิดการกลายพันธุ์และอาการทางคลินิก มีความแตกต่างกันไปในระหว่างกลุ่มเชื้อชาติ สำหรับในประเทศไทยความหลากหลายของยีน TTR ยังไม่เคยมีปรากฏรายงานมาก่อน ดังนั้นในงานวิทยานิพนธ์ครั้งนี้จึงได้ทำการศึกษาความหลากหลายของยีนและค้นหา ApoE และ TTR ในกลุ่มคนไทยที่มีความเสี่ยงสูงต่อการเกิดโรคอัลไซเมอร์ ซึ่งในการศึกษาจีโนไทป์ของยีน ApoE โดยวิธี Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) นั้นได้ทำการศึกษาจาก Genomic DNA ที่เตรียมได้จากกลุ่มคนปกติจำนวน 48 ราย (อายุเฉลี่ย 60$\pm$ ปี), กลุ่มผู้ป่วยโรค Down's syndrome (อายุเฉลี่ย 12.64 $\pm$ 5.57 ปี เป็นเพศชาย 19 ราย และ เพศหญิง 14 ราย), กลุ่มผู้ป่วยที่ไม่ใช่ผู้ป่วย Down's syndrome(DS) แต่มีความเสี่ยงสูงต่อการเกิดโรคอัลไซเมอร์ (other Alzheimer Disease High Risk; ADHR) จำนวน 55 ราย (อายุเฉลี่ย 46.78 $\pm$ 11.45 ปี เป็นเพศชาย 19 ราย และ เพศหญิง 36 ราย) และกลุ่ม

ควบคุมของกลุ่ม ADHR จำนวน 101 ราย (อายุเฉลี่ย 45.31±10.81 ปี เป็นเพศชาย 31 ราย และ เพศหญิง 70 ราย)

จากการศึกษาทั้ง 4 กลุ่มตัวอย่างชี้ให้เห็นว่า ε3/ε3 เป็นจีโนไทป์ที่พบมากใน ทั้ง 4 กลุ่ม สอดคล้องกับที่เคยมีการรายงานมาแล้วในกลุ่มคนสุขภาพดีและกลุ่มผู้ป่วยโรค Down's syndrome ที่มีอายุในเชื้อชาติอื่นๆ และยังพบอัลลีล ε3 มากในทั้ง 4 กลุ่มตัวอย่างอีก ด้วย การศึกษาในครั้งนี้ไม่พบ homozygous ε2/ε2 ในทั้ง 4 กลุ่มตัวอย่างซึ่งแตกต่างจากที่เคย มีรายงานในกลุ่มคนไทยที่มีสุขภาพดี แต่เหมือนกับรายงานที่เคยมีในการศึกษากลุ่มคนไทย สูงอายุทั้งที่เป็นและไม่เป็นโรคสมองเสื่อม ซึ่งจากรายงานในครั้งนั้นคาดว่า ε2/ε2 อาจมีบทบาท ในการขัดขวางการเกิดโรคสมองเสื่อมในกลุ่ม DS และกลุ่มสูงอายุ การศึกษาในวิทยานิพนธ์ครั้ง นี้ยังพบความถี่ของ homozygous ε4/ε4 ในกลุ่ม DS เป็น 3 เท่าของกลุ่มคนปกติ ซึ่งเมื่อ เปรียบเทียบแล้วแตกต่างจาก DS ในกลุ่มเชื้อชาติอื่นๆ ทำให้เชื่อได้ว่ากลุ่มคนไทยที่เป็นโรค DS นี้ จะมีการพัฒนาไปสู่โรคอัลไซเมอร์ได้เร็วกว่าในกลุ่มเชื้อชาติอื่นๆ เมื่อเปรียบเทียบกลุ่ม ADHR กับกลุ่มควบคุมของ ADHR พบว่าจีโนไทป์ ε2/ε3, ε3/ε3, ε2/ε4 และ อัลลีล ε2, ε3 มีความถี่ เช่นเดียวกับกลุ่มเชื้อชาติอื่นๆรวมทั้งยังเหมือนกับกลุ่มคนปกติและกลุ่ม DS อีกด้วย ส่วน ความถี่ของจีโนไทป์ ε3/ε4 , ε4/ε4 และอัลลีล ε4 ในกลุ่ม ADHR มีความแตกต่างจากกลุ่ม ควบคุมของ ADHR โดยพบความถี่ของ ε3/ε4 และ ε4/ε4 เป็น 3 เท่า และอัลลีล ε4 เป็น 2 เท่าของกลุ่มควบคุมของ ADHR คาดได้ว่ากลุ่ม ADHR นี้น่าจะมีความเสี่ยงสูงต่อการพัฒนาไป เป็นโรคสมองเสื่อมและโรคอัลไซเมอร์

จากการศึกษาการกลายพันธุ์ของยีน TTR บริเวณ exon1, exon2 และ exon4 ของกลุ่มตัวอย่างจำนวน 173 ราย และการศึกษายีน TTR บริเวณ exon1 และ exon4 ของ ตัวอย่าง AD paraffin-embedded โดยวิธี Single-Stranded Conformation Polymorphism (SSCP) พบความผิดปกติในการเคลื่อนที่ในกระแสไฟฟ้าของ TTR exon1 จำนวน 3 ราย และ TTR exon4 จำนวน 2 ราย ผลการศึกษาลำดับเบสของ TTR exon1 ที่ผิดปกตินั้น พบว่าเกิด การแทนที่เบส Cytosine โดย Guanine ซึ่งส่งผลให้เกิด Single Nucleotide Polymorphism (SNP) ใน intron1 ณ ลำดับนิวคลีโอไทด์ที่ 22 จากปลาย 3′ของ TTR exon1

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ABSTRACT

Amyloid β (Aβ) is the major component of senile plaques found in Alzheimer's disease (AD). An increase production of amyloid precursor protein (APP), which leads to high levels of Aβ and also mutation of some other amyloid related proteins is the major cause in formation of the amyloid plaque in organs particular brain. Therefore, people with some genetic disorder such as Down's symdrome (trisomy 21) who contain three copies of chromosome, thus, have high risk in developing the AD. Among proteins in plasma, apolipoprotein E (ApoE) and transthyretrin (TTR) are two of those important proteins that related to the amyloid plaque formation. The association of the ApoE gene alleles in particular ApoE-ε4 with AD has been supported and it is different among the ethic backgrounds. In Thailand, genotype of ApoE was reported in only normal and elderly groups (Senanarong *et al*., 2001). Similarly, genetic variation of TTR which is the main constituent of amyloid that deposits preferentially in peripheral nerve and leads to familial amyloid polyneuropathy (FAP), familial amyloid cardiomyopathy (FAC) and senile systemic amyloidosis, is also different among population. More than 80 mutations of TTR have been identified associated to the diseases with frequency and clinical manifestations vary among populations. In Thailand, the genetic variation of the TTR gene has never been reported. In this thesis, variation of ApoE and TTR genes was examined in Thai people with high risk in AD in order to search for and characterize the mutation of the genes. Genomic DNAs from 48 normal people (average age is over 60 years), 33 patients with DS (average age is 12.64 ± 5.57 years; 19 males and 14 females), 55 cases with other AD high risk (ADHR) (average age is 46.78 ± 11.45 years; 19 males

and 36 females) and 101 ADHR control (average age is 45.31±10.81 years; 31 males and 70 females) were prepared and used in study for ApoE genotype by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP).

The results indicated ε3/ε3 was the most common genotype found in all of these four Thai groups, similarly to that previous reported in healthy and elderly DS of other ethnics. In addition, ε3 was the most common allele. An absence of the homozygous ε2/ε2 in all Thai groups was observed with un-expectation, and it differed from that previously reported for Thai healthy however is similar to that found in Thai elderly with and without dementia. This might suggest the protective role in development of dementia in only DS and elderly. The frequency of homozygous ε4/ε4 was three times in Thai DS, different from the DS in other ethnics, convincing to faster development of AD in Thai DS. By comparing with control, the ADHR showed similar frequencies of ε2/ε3, ε3/ε3, ε2/ε4, ε2 and ε3 to other ethnics and also to the normal and the DS groups studied here. The frequency differences were only observed in ε3/ε4 and ε4/ε4 genotypes and the ε4 allele. The ε3/ε4 and ε4/ε4 were triple and the ε4 allele was double comparing to the control, suggesting to high risk in development of dementia and AD in the ADHR group.

Mutation of the TTR gene in exon1, exon2 and exon4 regions in 173 Thai people and that in exon1 and exon4 regions in the AD paraffin-embedded specimen were determined by Single-stranded conformation polymorphism (SSCP). Three and two samples showed abnormal electrophoresis mobility of the exon1 and exon4 fragments, respectively. According to nucleotide sequencing, it was revealed the abnormality in exon1 fragment was due to the C→G substitution single nucleotide polymorphism (SNP) in TTR intron1 at position 22 downstream to the 3′end of the TTR exon1.

ACKNOWLEDGEMENT

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LIST OFABBREVIATIONS AND SYMBOLS

A	=	absorbance
Å	=	angström
α	=	alpha
Aβ	=	amyloid β
AD	=	alzheimer's disease
AN	=	autonomic neuropathy
AOX	=	alcohol oxidase
Arg	=	arginine
ApoE	=	apolipoproteinE
APP	=	amyloid Precursor Protein
β	=	beta
bp	=	base pairs
BMGY	=	buffer medium containing glycerol
BMMY	=	buffer medium containing methanol
C	=	cytosine
CAA	=	cerebral amlyoid angiopathy
cDNA	=	complementary deoxyribonucleic acid
C-terminal	=	carboxyl-terminal
CSF	=	cerebrospinal Fluid
CTS	=	carpal tunnel syndrome
Δ	=	delta
DMSO	=	dimethylsulfoxide
DNA	=	deoxyribonucleic acid
dNTP	=	deoxynucleotide triphosphate
DTT	=	dithiothreitol
ε	=	epsilon
EDTA	=	ethylenediaminetetraacetate
ER	=	endoplasmic reticulum

LIST OFABBREVIATIONS AND SYMBOLS

(Continued)

F	=	phenylalanine
FAC	=	familial Amyloidotic Cardiomyopathy
FAP	=	familial Amyloidotic Polyneuropathy
G	=	gaunine
γ	=	gamma
Glu	=	glutamine
gp	=	glycoprotein
h	=	hour
HCl	=	hydrochloric acid
HDL	=	high-density lipoproteins
IDL	=	intermediate-density lipoprotein
I-DOX	=	4′-deoxy-4′-iodoxorubicin
kb	=	kilobase
kcal	=	kilocalories
Kcl	=	potassium chloride
kDa	=	kilodalton
L	=	localized
LB	=	luria-Bertani
LDL	=	low density lipoprotein
LDLR	=	low density lipoprotein receptor
Leu	=	leucine
LM	=	leptomenigeal amyloidosis
MD	=	minimal dextrose medium
mRNA	=	Messenger ribonucleic acid
mg	=	milligram
min	=	minute
ml	=	milliliter

LIST OFABBREVIATIONS AND SYMBOLS

(Continued)

M	=	molar
$MgCl_2$	=	magnesium chloride
mM	=	millimolar
MM	=	minimal methanol medium
N-terminal	=	amino-terminal
ng	=	nanogram
ºC	=	degree Celcius
OD	=	optical density
PAGE	=	polyacrylamide gel electrophoresis
PBS	=	phosphate buffer saline
PCR	=	polymerase chain reaction
%	=	percentage
pmol	=	picomole
PN	=	peripheral neuropathy
RFLP	=	restriction fragment length polymorphism
rpm	=	revolutions per minute
RBP	=	retinol binding protein
s	=	second
S	=	systemic
SDS	=	sodium dodecyl sulfate
SSA	=	senile systemic amyloidosis
SSCP	=	single-stranded conformation polymorphism
T3	=	3,5,3´-triiodo-L-thyronine
T4	=	L-thyroxine
Ta	=	annealing temperature
Taq	=	*thermus aquaticus*
TBE	=	tris-borate EDTA
TBG	=	thyroxine Binding Globulin

LIST OFABBREVIATIONS AND SYMBOLS

(Continued)

TTR	=	transthyretin
Trp	=	typtrophan
UK	=	United Kingdom
Val	=	valine
VLDL	=	very low density lipoprotein
V30M	=	valine30methionine
V122I	=	valine122isoleucine
YPD	=	yeast extract peptone dextrose
v/v	=	volume by volume
wt	=	wild-type
μF	=	micro Faraday
μg	=	microgram
μl	=	microliter
μM	=	micromolar

CHAPTER 1

INTRODUCTION

Introduction

Amyloid β (Aβ) is the major component of senile plaques found in Alzheimer's disease (AD).The Aβ also exists in a soluble form in physiological fluids such as cerebrospinal fluid (CSF) and plasma as well as in the conditioned media of different cultured cell lines (Smith and Anderton, 1994; Haass *et al.*, 1992; Seubert *et al.*, 1992). The mechanism by which Aβ forms the fibrils is unclear. But several evidences suggested that the fibril formation is a critical step in the pathogenesis of AD. CSF contains several factors that promote the solubility, transport and clearance of Aβ,mainly, two important proteins are apolipoprotein E (ApoE) and transthyretrin (TTR).

Several evidences pointed to an active role of ApoE and TTR in fibril formation in the brain. ApoE binds to β-peptide with high affinity *in vitro* (Strittmatter *et al.*, 1993), promotes Aβ fibrillogenesis *in vitro* and induces accumulation of the β-amyloid peptide in cultured vascular smooth muscle (Ma *et al.*, 1994; Wisniewski *et al.*, 1994; Mazur-Koleka *et al.*, 1995), whereas, TTR inhibits and prevents this fibril formation (Mazur-Koleka *et al.*, 1995; Schwarzman *et al.*, 1994). TTR in CSF was shown negative correlation with the degree of dementia in the patients with AD, while it has been shown associated with ApoE in AD. The ApoE levels increased in lumbar CSF in cases with normal aging and more extensively with AD (Merched *et al.*, 1997; Lindh *et al.*, 1997; Song *et al.*, 1997). The TTR levels also increased with aging but decreased with AD.

The AD was first discovered by Alois Alzheimer. This disease occurs mainly after age 65 and is the most common cause of dementia in the elderly. Memory loss, mainly the recent memory, and impaired judgment are the classic symptoms of AD. Normally the nerve cells in brain are arranged in an orderly manner. In persons with AD the cells are extremely disorganized and dysfunctional. As the brain cells stop working, part of the brain dies. This is the cause of memory loss. On

chromosome 21 of human, the gene coding for the amyoid precursor protein (APP), which is a precursor of the Aβ, is located. A genetic fault in this gene was identified as the first theorized cause of AD. People with DS are trisomy, therefore the patients have three instead of two sets of this gene in cell, leading to high production of APP and, in the consequence, high levels of the Aβ peptide. This is the reason why some people with DS develop AD at unusually younger ages. With build up of amyloid protein, the presence of neuritic plaques and neurofibrillary tangles is also seen in people with DS.

Among populations, association of the ApoE-ε4 allele with AD was different among ethnic background. In Caucasians, the ApoE allele is a risk factor of cerebral amlyoid angiopathy (CAA) and it would be independent of AD (Mazur-Koleka *et al.*, 1995; Schwarzman *et al.*, 1994). Whereas, in Japanese, the strong association existing between ApoE-ε4 allele and AD (Serot *et al.*, 1994). In addition, mutation in ApoE gene has been recently reported (Kambo *et al.*, 1999; Scacchi *et al.*, 2003), and higher risk of developing AD was associated with the mutation (Scacchi *et al.*, 2003). In Thailand, genotype of ApoE was reported in only 2 groups of people, normal and elderly people. Distribution of ApoE genotype in the elderly people was similar to that reported in other ethic groups (Senanarong *et al.*, 2001), and with bearing of ApoE-ε4 allele to the increase of the risk of developing dementia.

In persons with DS, a large variation of ApoE allele frequencies has been found (Anello *et al.*, 2001; Royston *et al.*, 1994 ; van Gool *et al.*, 1995), most likely reflecting the different geographic origins. The relation between ApoE-ε4 and the risk and onset of AD has been controversial. Some studies showed an increased risk of AD associated with the ApoE-ε4 allele in persons with DS (Deb *et al.*, 2000; Prasher *et al.*, 1997; Schupf and Segievsky, 2002), but others could not confirm this (Anello *et al.*, 2001; Lucarelli *et al.*, 2003; van Gool *et al.*, 1995). There is evidence that ApoE-ε2 is associated with increased longevity in some studies of DS (Prasher, 1992; Prasher *et al.*, 1997; Tyrrell *et al.*, 1998), but a protective effect on AD (Lai *et al.*, 1999; Rubinsztein *et al.*, 1999; Tyrrell *et al.*, 1998) could not be confirm by all (Deb *et al.*, 2000). The ApoE-ε4 allele has been associated further with early mortality (Folin *et al.*, 2003), but a meta-analysis based on data from 538 persons with DS

showed no significant evidence for an increased mortality of ApoE-ε4 homozygotes with DS (Edland *et al*., 1997). From biochemical perspective, one may assume that ApoE-ε4 is involved in dementia through the amyloid pathway (Lott and Head, 2005; Margallo-Lana *et al*., 2004). ApoE-ε4 may interact with the Aβ peptide to increase aggregation of the Aβ. However, on the assumption that having three copies of APP may overwhelm the effect of the ApoE-ε4 on Aβ metabolism, possible make the ApoE genotype less relevant for persons with DS in comparison to the general population.

Similarly to ApoE, genetic variation of TTR gene among populations has been reported. TTR Val30Met (V30M), is a major cause of familial amyloid polyneuropathy (FAP) in Portuguess (Saraiva *et al*., 1995), Swedish (Andersson *et al*., 1976) and Japanese (Araki *et al*., 1980), whereas, the Val122Ile (V122I) variant, carried by 3.9% of the African-Americans, is the most common cause of familial amyloid cardiomyopathy (FAC) (Jacobson *et al*., 1992). In French, up to 10 point mutations of the TTR gene have been identified so far and all led to development of the same pattern of neuropathy (Planté-Bordeneuve *et al*., 1998). Thus, information of the genetic variation is essential for an effective diagnosis and specifically treatment of the patient group.

TTR has been demonstrated as the most abundant protein component of amyloid fibrils (Costa *et al*., 1978) that lead to a group of diseases called amyloidosis. Typical manifestations of the disease caused by deposition of the TTR amyloids are peripheral neuropathy, cardiomyopathy, carpal tunnel syndrome, and vitreous opacities. Up to date, over 100 genetic TTR variants derived from a single amino acid substitutions have been reported (for review, sees Schwarzman *et al*., 2004) and up to 80 are related to amyloidosis with most clinical syndromes, familial amyloidotic polyneuropathy (FAP) that TTR amyloids deposition is systemically in the peripheral nervous system, and familial amyloidosis cardiomyopathy (FAC). In addition, the native TTR with an entirely normal amino acid sequence can also find in patients with senile systemic amyloidosis (Westermark *et al*., 1990).

FAP and FAC were described as the autosomal dominant inherited diseases caused by different single point mutations of the TTR gene. However, role of TTR mutations in the amyloidogenesis is not well understood and different mutations

are often associated with distinct clinical symptoms. Several reports suggested that point mutations of TTR gene could change conformation of the protein to an intermediate oligomer with an extensive β-sheet. Without a proper therapy, TTR amyloidosis can result in the fatal course, leading to death within 10 to 15 years. Orthopic liver transplantation is the most powerful therapy available nowadays. The treatment was shown to virtually eliminate TTR variant from plasma of the patients with FAP. However, it does not provide a practical means of treatment for a large number of patients. Moreover, the cardiac amyloidosis could progress and its effect on autonomic dysfunction has not been established (Adams *et al.*, 2000). Thus, other therapeutic strategies, e.g. a specific blocking an expression of the mutant TTR gene and stabilization of native conformation of the TTR with chemicals such as 4′-deoxy-4′-iodoxorubicin (IDOX), tetracycline and nitrophenols (Cardoso *et al.*, 2003) are being pursued. Frequency and clinical manifestations including age of onset, sex ratio and duration of the disease generated by deposition of the TTR amyloids are varied among populations. For examples, among the pathogenic TTR identified so far, V30M is the most frequent variant (Saraiva, 1984, 2001), vastly found in Portuguese and Swedish. In contrast, heterogeneity of the point mutations of TTR that result in more than 20 different amino acid substitutions including V30M has been reported among Japanese (Andersson, 1970; Araki, 1984; Ikeda *et al.*, 2002). Similar to that observed in French (Planté-Bordeneuve *et al.*, 1998), heterogeneity incident of Leu55Pro (L55P) and V122I on FAC was reported in many other populations including Taiwanese and African Americans (Jacobson *et al.*, 1992; Yamamoto *et al.*, 1994), putting these people to a significant risk for congestive heart failure. In Thailand, no genetic variation of the TTR gene has been studied so far. To accomplish for direct diagnosis and specific treatment for Thai patients, information on type and characteristics of TTR variant distribute in the area is essential.

Review of Literatures

1.   ApolipoproteinE (ApoE)

   1.1 General

ApoE is a glycoprotein that is important in lipid storage, transport, and metabolism (Mahley, 1988). It is mainly synthesized in liver, but also found in peripheral and central nervous systems, including of brain. ApoE has long been of interest in medicine, but its importance in neuroscience has increased dramatically since the identification of the ε4 allele of the ApoE gene as a major risk factor for the development of late-onset AD in elderly (Saunders *et al*., 1993; Strittmatter *et al*., 1993). This discovery led to a growing number of studies examining the role of the ApoE gene in normal brain function and cognition, as well as in disorders such as AD, brain injury, and stroke (Higgins *et al*., 1997; Horsburgh *et al*., 2000). Polymorphisms of the ApoE gene are associated with significant alterations in brain morphology (Plassman *et al.,* 1997) and cognitive functioning, including attention (Greenwood *et al*., 2000) and memory (Bondi *et al*., 1995). Studies of ApoE thus are focused with aim to reveal information relevant to the genetics of attention and memory in normal individuals. At the same time, such studies may identify cognitive and neural changes that may be characteristic of preclinical stages of AD.

Genetically, the ApoE-ε4 allele is associated with both familial late onset and sporadic AD and atherosclerosis (Greenow *et al*., 2005; Lane and Farlow, 2005; Tanzi and Bertram, 2005). AD patients carrying the ApoE-ε4 allele have more profound deposition of Aβ in their brains than those carrying other ApoE alleles (de la Torre, 2002; Farrer *et al*., 1997). Considerable evidence supports that ApoE4 increases the risk of AD by accelerating the plaque formation and by impairing the neurons. ApoE4 appears to modulate processing of APP and production of Aβ through both the LDL receptor-related protein pathway and domain interaction (Ye *et al*., 2005). Strong correlation of ApoE-ε4 allele with dyslipidemia and atherosclerosis, the major underlying mechanism of coronary heart disease, has been demonstrated (Gregg and Brewer, 1998). Human ApoE4 represents a dual risk factor for these two major degenerative diseases.

NH₂-Lys-Val-Glu-Gln-Ala-Val-Glu-Thr-Glu-Pro-Glu-Pro-Glu-Leu-Arg-Gln-Gln-Thr-Glu-Trp-Gln-Ser-Gly-Gln-Arg-Trp-Glu-Leu-Ala-Leu-Gly-Arg-Phe-Trp-Asp-Tyr-Leu-Arg-Trp-Val-Gln-Thr-Leu-Ser-Glu-Gln-Val-Gln-Glu-Glu-Leu-Leu-Ser-Ser-Gln-Val-Thr-Gln-Glu-Leu-Arg-Ala-Leu-Met-Asp-Glu-Thr-Met-Lys-Glu-Leu-Lys-Ala-Tyr-Lys-Ser-Glu-Leu-Glu-Glu-Gln-Leu-Thr-Pro-Val-Ala-Glu-Glu-Thr-Arg-Ala-Arg-Leu-Ser-Lys-Glu-Leu-Gln-Ala-Ala-Gln-Ala-Arg-Leu-Gly-Ala-Asp-Met-Glu-Asp-Val-`Cys`-Gly-Arg-Leu-Val-Gln-Tyr-Arg-Gly-Glu-Val-Gln-Ala-Met-Leu-Gly-Gln-Ser-Thr-Glu-Glu-Leu-Arg-Val-Arg-Leu-Ala-Ser-His-Leu-Arg-Lys-Leu-Arg-Lys-Arg-Leu-Leu-Arg-Asp-Ala-Asp-Asp-Leu-Gln-Lys-`Arg`-Leu-Ala-Val-Tyr-Gln-Ala-Gly-Ala-Arg-Glu-Gly-Ala-Glu-Arg-Gly-Leu-Ser-Ala-Ile-Arg-Glu-Arg-Leu-Gly-Pro-Leu-Val-Glu-Gln-Gly-Arg-Val-Arg-Ala-Ala-Thr-Val-Gly-Ser-Leu-Ala-Gly-Gln-Pro-Leu-Gln-Glu-Arg-Ala-Gln-Ala-Trp-Gly-Glu-Arg-Leu-Arg-Ala-Arg-Met-Glu-Glu-Met-Gly-Ser-Arg-Thr-Arg-Asp-Arg-Leu-Asp-Glu-Val-Lys-Glu-Gln-Val-Ala-Glu-Val-Arg-Ala-Lys-Leu-Glu-Glu-Gln-Ala-Gln-Gln-Ile-Arg-Leu-Gln-Ala-Glu-Ala-Phe-Gln-Ala-Arg-Leu-Lys-Ser-Trp-Phe-Glu-Pro-Leu-Val-Glu-Ash-Met-Gln-Arg-Gln-Trp-Ala-Gly-Leu-Val-Glu-Lys-Val-Gln-Ala-Ala-Val-Gly-Thr-Ser-Ala-Ala-Pro-Val-Pro-Ser-Asp-Asn-His-**COOH**

Figure 1.1   Amino acid sequence of human ApoE3 (wildtype) isoform (Cys112/Arg 158)

1.2 General structure and chemical properties of human ApoE

Human ApoE is a protein consisting of 299 amino acid residues (Figure 1.1) with a molecular mass of 34 kDa. It serves as a ligand for the low density lipoprotein (LDL) receptor family, and through this interaction, plays a major role in modulating plasma lipoprotein metabolism (Mahley, 1988; Weisgraber, 1994). ApoE is polymorphic with three major isoforms (ApoE2, ApoE3, and ApoE4) and a number of rare variants (Mahley, 1988; Rall & Mahley, 1992; Weisgraber, 1994). Majority of the ApoE isoforms differ at positions 112 and 158. ApoE3 contains cysteine and arginine at those two positions respectively, while ApoE2 contains cysteine and ApoE4 contains arginine at both positions (Weisgraber *et al*., 1981) (Figure 1.2). This polymorphism has functional consequences. ApoE2 binds defectively to the LDL receptor, resulting in type III hyperlipoproteinemia, which is a lipid disorder associated with premature heart disease (Mahley & Rall, 1995). This defective binding results from the substitution of arginine by cysteine at position 158 and is mediated indirectly, through rearrangement of salt bridges (Wilson *et al*., 1994; Dong *et al*., 1996). ApoE4 is associated with increased lipid levels and an increased risk of cardiovascular disease (Utermann *et al*., 1984; Eichner *et al*., 1993; Luc *et al*., 1994). The allele is also a major risk factor for AD (Corder *et al*., 1993; Saunders *et al*., 1993; Strittmatter *et al*., 1993) and other forms of neurodegeneration (Mayeux *et al*., 1995; Slooter *et al*., 1997; Teasdale *et al*., 1997).

ApoE is composed of two functionally distinct domains (Aggerbeck *et al*., 1988; Wetterau *et al*., 1988). Thrombin cleavage of ApoE results in a 22 kDa amino-terminal domain (residues 1–191) and a shorter 10 kDa carboxy-terminal fragment (residues 216–299) (Figure 1.3 A, B) (Innerarity *et al*., 1983). The region spanning from residues 136-160 of the 22 kDa (N-terminal) domain is rich in basic amino acids and is involved in LDL receptor binding (Innerarity *et al*., 1983; Dyer and Curtiss, 1991; Weisgraber, 1994 and Siest *et al*., 1995) (Figure 1.3 B) (Innerarity *et al*., 1983; Dyer and Curtiss, 1991; Weisgraber, 1994 and Siest *et al*., 1995). The 10 kDa (C-terminal) fragment carries the major lipid-binding determinants of ApoE (Weisgraber, 1990) and is responsible for the tetramer forming of the intact ApoE protein (Aggerbeck *et al*., 1988). The C-terminal domain, which is predicted to contain three helices, is less well known. The first two helices, comprising residues

203-223 and residues 225-266, are class A type, and the third helix, consisting of residues 268-289, is a G* helix (Nolte and Atkinson, 1992). The G* helix and part of the end of the second helix is expected play a key role in lipid binding and lipid interaction in ApoE-containing lipoproteins (Sparrow *et al*., 1992) (Figure 1.3 B)

The ApoE4 binds preferentially to VLDL and the interaction between Arg-61 and Glu-255 was suggested stabilize an extended helical structure in the carboxyl terminus to accommodate a larger less-curved VLDL surface (Dong and Weisgraber, 1996). The C-terminal domain of ApoE beyond residue 191, i.e. residues 203-223, 225-266, and 268-289, contains three predicted helices (Figure 1.3 A) (Wilson *et al*., 1991; De Pauw *et al*., 1997). The end of the second helix was proposed play a key role in lipid binding in ApoE and lipoprotein interaction (Westerlund and Weisgraber, 1993; Weisgraber, 1994). The third helix (G* helix) (Figure 1.3 B) induces the aggregation of C-terminus that becomes monomer after the polar/charged mutation of five amino acid residues, i.e. Phe257, Trp264, Val269, Leu279, and Val287 (Fan *et al*., 2004). Choy *et al*. (2004) have also shown intermolecular coiled-coil helical formation in the C-terminal domain. The C-terminal region of ApoE was also suggested interact with Aβ in forming neurofibrillary tangle (Huang *et al*., 2001). ApoE4 is one of the major proteins associated with Aβ plaques (Wisniewski *et al*., 1994, Puglielli *et al*., 2003). The role of ApoE in the molecular pathogenesis of AD might be related to its isoform-specific interactions with lipids or Aβ aggregates. However, the structural characters of ApoE isoforms that may lead to the differences in molecular pathogenesis of AD remain poorly understood.

Like other soluble apolipoproteins, ApoE is stable in both lipid associated and lipid-free aqueous states. The helices in the four helix bundle of ApoE are amphipathic, classified as G* by Segrest *et al*. (1994) (Figure 1.3 B), placing them between the typical globular amphipathic helices and the helices typical of other apolipoproteins. The amphipathic nature and particular character of the apolipoprotein helices are thought to be responsible for their lipid-binding properties (Segrest *et al*., 1994). It has been hypothesized that the amino-terminal four-helix bundle undergoes a significant conformational change, opening to expose the hydrophobic core to interact with lipid (Weisgraber, 1994). Molecular area measurements at an air:water interface support this model (Weisgraber, 1994). Although the major lipid-binding elements of

ApoE are contained in the C-terminal domain, the 22 kDa (N-terminal) domain, the 10 kDa (C-terminal) fragment, and the intact protein will remodel spherical vesicles of dimyristoylphosphatidylcholine (DMPC) and other phospholipids into bilayer discoidal particles (Innerarity *et al*., 1979, 1983). However, the conformation of ApoE on these phospholipid discs is still unknown. Two models have been proposed: the "picket fence" model, in which the amphipathic helices are aligned parallel to the phospholipid acyl chains (De Pauw *et al*., 1995), and the "belt" model, in which the helices lie perpendicular to the acyl chains (Raussens *et al*., 1998). In addition to its roles in cardiovascular and neurodegenerative diseases, ApoE has been implicated in immuno-regulation (Hui *et al*., 1980; Avila *et al*., 1982), intracellular cholesterol metabolism modulation (Reyland and Williams, 1991), and cell growth and migration control (Ishigami *et al*., 1998). These diverse roles underscore the importance of ApoE as a potential target for therapeutic agents and highlight the need for a better understanding of the relationship of its structure and function.
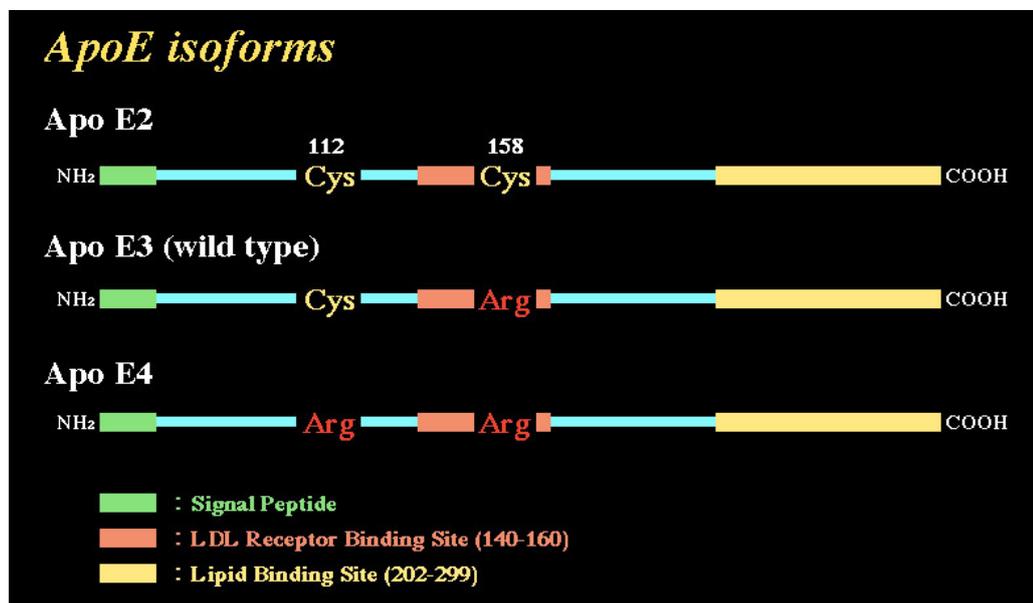
Figure 1.2   Structure of ApoE isoforms

There are three isoforms of ApoE by which differ from each other only a single amino acid substitution at residues 112 and 158.

ApoE2 (Cys112/Cys158), ApoE3 (wildtype; Cys112/Arg158), and ApoE4 (Arg112/Arg158).

A

C-terminal 10-kDa Domain

N-terminal 22-kDa Domain

Major Lipid Binding Region

N

112

158

LDL Receptor Binding Region

B

Residue number

1   50   100   150   200

ApoE   N   C

Helix   1   2   3   4   4-helix bundle

Class G* amphipathic helix   Class A amphipathic helix

136 — 160 192 — 299

Receptor-binding region   Lipid-binding region

1 — 191   244 — 272

Aβ aggregation-inhibiting region   Aβ-binding region

216 — 299

Region co-purified with Aβ from senile plaques

Figure 1.3   **(A) Three dimensional structure (3D) of human ApoE**

Two domains of ApoE, N-terminal and C-terminal domains, are shown in figure. The N-terminal domain contains four helices that arrange into anti-parallel elongated bundles, while the C-terminal domain contains three helices. The LDL receptor binding region in the N-terminal domain, and the major lipid-binding site in the C-terminal also are shown.

(Source: Chou *et al.*, 2006)

(B) Structures of class A and class G* amphipathic helices.

The class A amphipathic helix structure of the first two helices, i.e. residues 203-233 and residues 225-266, and the class G* helix structure of the third helix, residues 268-289, of ApoE C-terminal domain are shown. The Aβ binding and inhibitory regions are also shown.

(Source: for review, sees Tomiyama *et al.*, 1999)

1.3 Gene structure of human ApoE

Apolipoprotein E (ApoE) is a component of various classes of plasma lipoproteins in all mammals (Mahley *et al*., 1979 and 1984). It is a single chain polypeptide (MW. 34,000 kDa) of 299 amino acids (Rall *et al*., 1982) that is initially synthesized with an 18-residue signal peptide that is co-translationally removed later (Paik *et al*., 1985). The amino acid and the mRNA nucleotide sequences are known for both in human (Rall *et al*., 1982; McLean *et al*., 1984) and rat (McLean *et al*., 1983). The major site of synthesis is liver, but relatively abundant levels of ApoE mRNA have been detected in many extrahepatic tissues, including brain and adrenals (Elshourbagy *et al*., 1985).

The gene coding for ApoE locates on chromosome 19 (19q13.2), consisting of four exons and three introns (Figure 1.4) (McLean *et al*., 1984). All introns begin with the nucleotides GT and end with the nucleotides AG, which is consistent with the consensus sequence for exon-intron splice junctions for eukaryotic genes (Breathnach *et al*., 1978). Lengths of the exons are 44, 66, 193, and 860 nucleotides, and that of the introns are 760, 1092, and 582 nucleotides (McLean *et al*., 1984). The overall ApoE gene is 3597 nucleotides, which encodes mRNA of 1163 nucleotides.

The nucleotide sequence of the 5' flanking region of the ApoE gene adjacent to the transcription initiation site revealed several potentially important sequence elements. It contains the sequence T-A-T-A-A-T-T (Figure 1.5) that is homologous to the "TATA box" sequence has been identified as a component of the promoter region for most eukaryotic genes (Paik *et al*., 1985). In addition, two major inverted repeated sequences are located within 150 nucleotides adjacent to the mRNA start site. The proximal element is located between nucleotides -76 and -46, and the distal element is located between nucleotides -144 and -108. Large number of GC base pairs in both proximal and distal elements sequences suggested these palindrome-like structures are the stable naturally occurring elements (Figure 1.5).

In the introns and the proximal flanking regions of the ApoE gene, there are four segments of Alu repeated sequences ranging from 280 to 324 nucleotides. Two of these sequences are located in the second intron, while another two locate in the non-transcribed flanking regions closely to both ends of the gene

(Figure 1.6). Upon alignment for maximum homology of nucleotide sequence, individual Alu sequences show 81%-90% of identity (Paik *et al*., 1985).

15
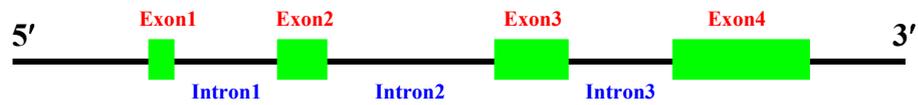
Figure 1.4   **The ApoE gene structure**

Gene of ApoE comprises 4 exons and 3 introns. Close boxes with Exon1, Exon2, Exon3 and Exon4 on top are ApoE exon 1, 2, 3 and 4, respectively. Line with Intron1, Intron2 and Intron3 underneath are ApoE intron1, 2 and 3, respectively.

Figure 1.5   Transcription initiation site and 5' flanking region of the ApoE gene.

Nucleotide sequences of the proximal 5' region adjacent to the transcription initiation site (A) and of the first exon (B) of the ApoE gene. Numbers indicate nucleotide positions relative to the initiation site (position 1). Bar indicates the TATA box. Inverted repeated sequences are shown with all potential base pairs. G-T base pairs indicated by a colon.

(Source: Paik *et al.*, 1985)

Figure 1.6   Alu family sequences in the human ApoE gene.

The ApoE gene with relative positions of exons (solid boxes, upper line) and Alu sequences (shaded boxes, lower line) are indicated. Nucleotide sequence positions of the first and last nucleotide of each element relative to transcription initiation site (position 1) are shown above the lines. Lengths of sequence elements are shown below the lines. Arrows show orientation of the Alu sequences relative to the coding strand of the ApoE gene. Roman numerals indicate the individual Alu sequence family members.

(Source: Paik *et al.*, 1985).

1.4 Role of ApoE

1.4.1   ApoE in plasma

Plasma lipoproteins are usually divided into five groups i.e. chylomicrons, very-low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL). Chylomicrons, the largest lipoproteins synthesized in the small intestine, transport dietary cholesterol and triglyceride to the liver and peripheral tissues. VLDLs, the second largest lipoproteins, are synthesized in the liver and secreted to distribute triglycerides to peripheral tissues. VLDLs are also metabolized by lipoprotein lipase into the remnant lipoprotein, IDL. The biological role of IDL is unclear. Most IDLs are further metabolized by lipase and produce the smaller cholesterol-rich lipoproteins, LDLs. LDLs, in turn, transport liver-synthesized cholesterol to peripheral tissues. In peripheral tissues, as well as in the liver, LDL binds to the LDL receptor and is taken up by endocytosis. HDLs, the smallest lipoproteins synthesized in the liver and the small intestine and also produced in the metabolic pathway including chylomicrons and VLDLs, participate in reverse cholesterol transport from peripheral tissues to the liver (for review, sees Beffert *et al*., 2004).

Each lipoprotein contains several specific apolipoproteins, such as ApoA (AI, AII and AIV), ApoB, ApoC (CI, CII and CIII) and ApoE. ApoE is a constituent of chylomicrons, VLDLs, IDLs and a subclass of HDLs, but not LDLs. It functions as a ligand for several lipoprotein receptors. Such as, the chylomicron remnant that contains the remaining triglyceride and cholesterol binds to ApoE receptor(s) in the liver and is taken up by receptor-mediated endocytosis. ApoE has multiple amphipathic helices, enabling it to interact with hydrophobic lipid particles and provide them with the hydrophilicity required for their transport in the blood (Weisgraber, 1994; Segrest *et al*; 1994). The C-terminus of ApoE was proposed to be a major lipid binding region (Figure 1.3 A) (Weisgraber, 1994; Segrest *et al*; 1994). Due to its hydrophobicity, the recombinant C-terminal fragment of ApoE tends to aggregate into amyloid-like fibrils (Wisniewski *et al*., 1995). However, ApoE does not exist as a free form in biological fluids.

1.4.2 ApoE in brain

ApoE is a ligand for all members of the LDL receptor (LDLR) family (for review, sees Beffert *et al.*, 2004) and a constituent of lipoprotein particles that transport lipids throughout the circulation and between cells. In the nervous system, non-neuronal cell types, most are astroglia and microglia, are the primary producers of ApoE, while neurons preferentially express the receptors for ApoE. Amongst the ApoE isoforms, the ε4 is at greater risk for developing coronary artery disease and late-onset AD, a devastating age related neurodegenerative disorder (Corder *et al.*, 1993). However, precisely how ApoE isoforms differentially affect an individual's risk for developing AD is under intense debate. Proposed mechanisms include roles for ApoE in cholesterol transport and synapse formation, modulation of neurite outgrowth and synaptic plasticity, destabilization of microtubules, amyloid clearance and fibril formation by direct binding of the Aβ peptide, and impairment of ApoE receptor-dependent protective signals that promote neuronal survival and synaptic plasticity.

ApoE is present in abundance in the brain as well as in the CSF. Lipid-associated ApoE binds to LDL receptor family members on the neuronal cell surface where it has been shown to modulate neurite outgrowth in an isoform-specific manner (Nathan *et al.*, 1994; Nathan *et al.*, 1995). ApoE-ε2 and ApoE-ε3 isoforms induce neurite extension in neurons, whereas ApoE-ε4 inhibits outgrowth. Blockade of ApoE receptors with the receptor-associated protein eliminates this effect on neurite extension (Holtzman *et al.*, 1995). Furthermore, strong *in vitro* synapse formation in neuronal cultures is dependent on glia-derived ApoE-cholesterol complex (Mauch *et al.*, 2001). These lead to hypothesize that neuronal plasticity is modulated by transporting lipid into neurons through specific receptors (Figure 1.7).

A hallmark of AD is the deposition of Aβ, which derives from the APP by sequential proteolytic cleavage, in senile plaques in the brain (Hardy and Allsop, 1991). The ultimate processing step involves intra-membranous cleavage by the presenilins and is affected by the cholesterol content of the membrane. Depletion of cellular cholesterol reduces the formation of neurotoxic Aβ protein (Simons *et al.*,

1998). ApoE can also bind Aβ directly (Strittmatter *et al*., 1993), thereby possibly contributing to its clearance and degradation in a process that may involve lipoprotein receptors (Rebeck *et al*., 1993). In mice, plaque formation is dependent on the presence of ApoE (Bales *et al*., 1997) and can be influenced by reintroducting human ApoE (Bales *et al*., 1999; Holtzman *et al*., 1999).
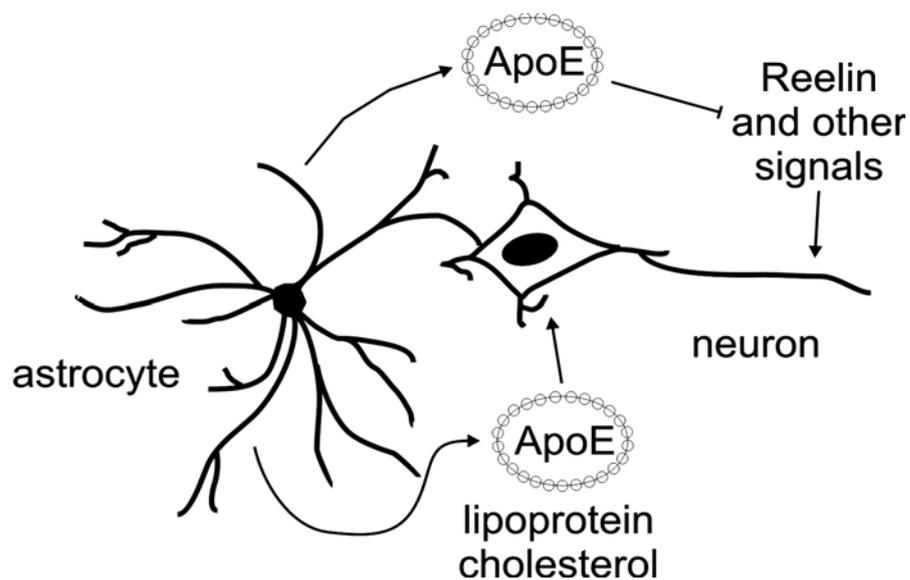
Figure 1.7   Physiological and hypothetical roles of ApoE in brain

ApoE is secreted by glial cells (Boyles *et al*., 1985; Boyles *et al*., 1989), and ApoE receptors are abundantly expressed on neurons. ApoE secretion by the glia serves, on one hand, to supply the neurons with the cholesterol necessary for synapse formation, thereby liberating considerable metabolic and biosynthetic capacity for other functions. This may have contributed to the rapid increase of brain size during the evolution of mammals. On the other hand, however, ApoE may also interfere with other important functions that are routed through the same receptors. The Reelin signaling pathway is likely just one example. Functional impairment of Reelin signaling would negatively impact synaptic plasticity and thus potentially adversely affect neuronal survival.

(Source: for review, sees Beffert *et al*., 2004)

1.5 ApoE genotype and late-onset AD

AD is a progressive, neurodegenerative disorder with the principal clinical symptom of dementia. It is characterized by three major pathological changes in brain, particularly in cerebral cortex and hippocampus. These are neuronal loss, senile plaques and neurofibrillary tangles. Senile plaques consist of extracellular amyloid deposits surrounded by dystrophic neurites, activated microglia and activated astrocytes. The principal component of this amyloid is Aβ. Neurofibrillary tangles, which accumulate in the cytoplasm of degenerating neurons, are composed of insoluble twisted filaments of abnormally phosphoryrated tau protein. Senile plaque formation is observed only in AD, DS and normal aging, whereas neurofibrillary tangles occur widely in numerous neurodegenerative disorders that are not accompanied by amyloid deposition. In DS, which features of neuropathology are indistinguishable from that of AD in younger ages, the deposition of amyloid has been shown to precede the appearance of neurofibrillary tangles and dementia. Thus, amyloid deposition has been thought more likely to be a cause of AD than neurofibrillary tangles.

In 1993, it was proposed that ApoE4 is a genetic risk factor for late-onset familial AD (Corder *et al*., 1993). The proportion of the late-onset AD increased from 20% with no copy of ε4 to 47% with one copy, and to 91% with two copies (Corder *et al*., 1993). In addition, the mean age of AD onset became younger as the ε4 alleles increased from 84.3 years with no copy to 75.5 years with one copy, and to 68.4 years with two copies (Corder *et al*., 1993). Not only familial but also sporadic late-onset AD was shown associated with the ε4 allele (Saunders *et al*., 1993). Moreover, gender difference in ApoE-associated risk for AD was also reported (Payami *et al*., 1996). A direct comparison of ε4 heterozygous between male and female revealed a significant twofold increased risk in women. However, in the contrary, ApoE-ε2 allele was demonstrated as a protective against late-onset AD (Corder *et al*., 1994).

The neuropathological relationship between ApoE and AD was first demonstrated in 1991 (Namba *et al*., 1991). The ApoE immunoreactivity to all cerebral and systemic amyloid types was observed (Wisniewski and Frangione, 1992).

At present, the ApoE allele and genotype are recognized as the most important genetic risk factor for late-onset AD (Corder *et al.*, 1998). In addition a study revealed the influences of ApoE-ε4 allele on pathogenesis of dementia and peripheral neuropathy in human immunodeficiency virus (HIV) infection, which elicits the inflammatory responses in the central nervous system (CNS) similar to those in AD (Corder *et al.*, 1998).

1.6 ApoE and Aβ in the pathogenesis of AD

Aβ is produced from its larger precursor APP (Selkoe, 1994), which is a glycoprotein with a single transmembrane domain and is expressed in both neural and non-neural tissues. The physiological role of APP is not well understood. Aβ is constitutively secreted as a soluble form during normal cellular metabolism and is detected in CSF and plasma of normal individuals as well as AD patients. There are two general pathways, α and β, for APP processing. In the α pathway, APP is cleaved within the Aβ sequence by α-secretase. In the β pathway, APP is sequentially cleaved at different sites in the extracellular and transmembrane domains by β-secretase and γ-secretase, respectively. Aβ is generated only in the β pathway as the predominant form of 40-amino acid peptide (Aβ1–40).

The amyloid cascade hypothesis, which was supported by the finding of neurotoxic of the exogenous Aβ peptides (Yankner *et al.*, 1990), is at present the dominant hypothesis concerning the pathogenesis of AD. A number of studies have demonstrated that the neurotoxicity of Aβ is dependent on its aggregation state (Pike *et al.*, 1993; Iversen *et al.*, 1995) and requires the assembly of Aβ into amyloid fibrils (Lorenzo and Yankner 1994). Length of the hydrophobic C-terminus of Aβ is critical in determining the rate of aggregation and the process of Aβ aggregation is a nucleation-dependent polymerization which can be accelerated by adding preformed aggregates as a seed (Jarrett and Lansbury 1993). Many evidences supported this hypothesis including predominantly deposition of the Aβ peptide ending at residue 42 (Aβ1–42) (Iwatsubo *et al.*, 1994) and alteration of APP processing to produce more Aβ1–42 in mutations in APP (chromosome 21), presenilin 1 (chromosome 14) and presenilin 2 (chromosome 1) in the early-onset familial AD (Hardy, 1997). According

to the hypothesis, aggregation of Aβ is enhanced by oxidation of Aβ (Dyrks *et al*., 1992) and in the presence of metal ions, such as Zn^{2+} (Bush *et al*., 1994). The Aβ spontaneously fragments and generates free radical peptides, which may react with one another to form aggregates and may attack nerve cell membranes (Hensley *et al*., 1994). The aggregated Aβ induces intracellular accumulation of reactive oxygen species (Behl *et al*., 1994) and disruption of cellular Ca^{2+} homeostasis in neurons (Mattson *et al*., 1992). The former effect was suggested to cause oxidative damage of neurons, probably via hydroxyl radical generation (Behl *et al*., 1994). Then, Aβ activates microglias (Meda *et al*., 1995), which in turn internalize microaggregates of Aβ via their scavenger receptors (El Khoury *et al*., 1996; Paresce *et al*., 1996), and are mobilized in pathological lesions and play role in inflammatory processes associated with amyloid plaques.

However, recently a few novel cascades of Aβ deposition have been proposed, including the lysosomal accumulation pathway and the plaque-trapping pathway. For the lysosomal accumulation pathway, the internalized ApoE induces intracellular accumulation of Aβ by inhibiting lysosomal enzymes and thereby alters APP metabolism, causing neuronal degeneration. Once neurons die, intracellular Aβ aggregates associated with ApoE become extracellular deposits (Figure 1.8 A). This cascade initiated from the fact that the internalized HDL particles containing ApoE:Aβ complexes that are thought to be delipidated in the endosomal:lysosomal pathway and ApoE and Aβ are expected to be degraded in lysosomes. However, it was found that the internalized Aβ was not completely degraded (Ida *et al*., 1996). Although most Aβ peptides were degraded after uptake, but small amounts of the peptide accumulated in insoluble fractions of the cells and remained stable for several days. Purified ApoE was shown to bind to Aβ with its hydrophobic C-terminal region (residues 244–272) *in vitro* (Strittmatter *et al*., 1993). Moreover, the C-terminal fragment residues 216–299 was shown to aggregate into amyloid-like fibrils *in vitro* and was co-purified with Aβ from senile plaques (Wisniewski *et al*., 1995). These led to speculate that the formation of ApoE:Aβ complexe is resistant to lysozyme.

It was shown that purified ApoE4 bound to Aβ more avidly than ApoE3 (Strittmatter *et al*., 1993). Although, ApoE3 rather ApoE4 induced

accumulation of Aβ in a higher percentage of cells, but the deposits induced by ApoE4 were more stable (Mazur-Kolecka *et al.*, 1995). Only cells containing Aβ deposits induced by ApoE4 exhibited decreased cellular redox activity (Mazur-Kolecka *et al.*, 1995). In AD brain, intracellular accumulation of ApoE was correlated with intracellular Aβ immuno-reactivity within the same cytoplasmic granules and with high expression of gp330: megalin, one of the ApoE receptors (Zheng *et al.*, 1994). DNA fragmentation was restricted to cells with intracellular Aβ immunoreactivity, but was not associated with extracellular Aβ deposition (LaFerla *et al.*, 1997). It is likely that the presence of Aβ1–42 facilitates intracellular aggregation:accumulation of Aβ and ApoE fragments, resulting in seeding for subsequent intracellular Aβ1–40 accumulation. In cultures of cells stably expressing APP, internalized Aβ1–42 aggregates induced intracellular accumulation of amyloidogenic C-terminal fragments of APP (Yang *et al.*, 1995). This abnormal accumulation of amyloidogenic C-terminal fragments of APP has been claimed to cause neuronal degeneration.

Plaque-trapping pathway is another possible pathway of ApoE-mediated Aβ deposition. In the pathway, ApoE-containing HDL should binds to Aβ1–42 deposits for the clearance process. However, because Aβ1–42 has a strong tendency to aggregate, HDL will be trapped by the plaques instead of removing Aβ peptides. The trapped HDL probably mobilizes soluble Aβ1–40 and Aβ1–42 peptides to the plaques through ApoE-Aβ interaction. The mobilized Aβ peptides are utilized for the growth of the plaques. Cholesterol within the trapped HDL may be removed by other, probably nascent HDL particles that contain little core lipid (LaDu *et al.*, 1998). Aβ deposition is thus accelerated by the presence of ApoE-containing HDL, and ApoE is incorporated into amyloid fibrils (figure 1.8, B). This hypothesis is supported by the finding that amyloid-associated ApoE purified from AD brain exists not as free molecules but as stable complexes with Aβ fibrils (Naslund *et al.*, 1995). However, the observation that immunoreactivity to ApoAI, another constituent of HDL, occasionally occurred in senile plaques (Wisniewski *et al.*, 1995) is not inconsistent with the hypothesis.
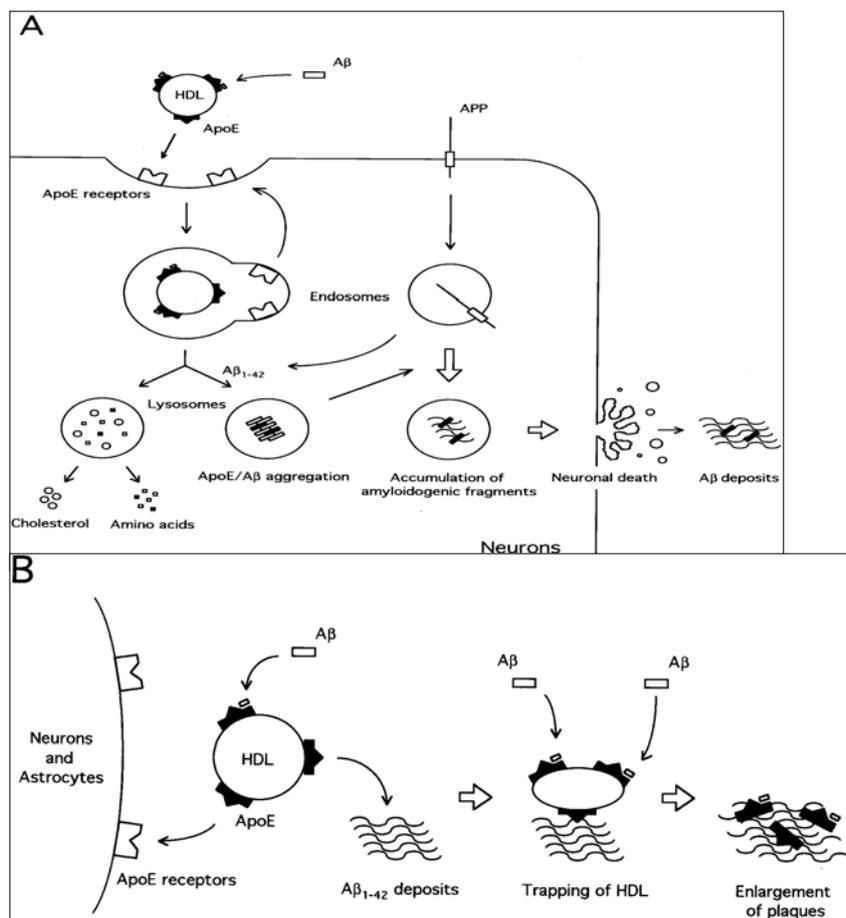
Figure 1.8   Models of ApoE-mediated Aβ deposition.

> **(A) The lysosomal accumulation pathway:** ApoE-containing HDL is internalized by neurons by receptor-mediated endocytosis. ApoE:Aβ complexes are resistant to lysosomal enzymes and thereby aggregate and accumulate intracellularly in which subsequently altered APP metabolism and thus neuronal degeneration. Once neurons die, intracellular Aβ aggregates become extracellular deposits. (Source: Tomiyama *et al.*, 1999)
>
> **(B) The plaque-trapping pathway:** ApoE-containing HDL binds to extracellular Aβ1–42 deposits to clear them, but is instead trapped by the plaques. The trapped HDL mobilizes soluble Aβ peptides (mainly Aβ1–40) to the plaques via ApoE:Aβ interaction, resulting in enhanced deposition of Aβ. (Source: Tomiyama *et al.*,1999)

2.   Transthyretin (TTR)

2.1 General

TTR is one of the three major thyroid hormone binding proteins found in the plasma of larger mammals. It was first found in both human serum and cerebrospinal fluid (CSF) (Kabat *et al*., 1942 a, b). The liver represents the main source of synthesis, while choroid plexus and retina produce only small amount of TTR. In addition to binding to thyroid hormones, both 3, 5, 3′, 5′-tetraiodothyronine (L-thyroxine or T4) and 3, 5, 3′-triiodothyronine (T3), TTR binds virtually all of serum retinol-binding protein (RBP). The general protein structure of TTR is a tetramer of identical subunits (Blake *et al*., 1978) with highly content of β-structure, which contributes to extraordinary stability of the molecule (Hanlon *et al*., 1971; Branch *et al*., 1972). Association of two dimers results in a tetramer structure with four of eight-stranded β-sheets.

2.2 Structure and chemical properties of TTR

TTR is a globular plasma protein comprising four identical subunits (Blake *et al*., 1978). It has a molecular mass of ~55 kDa and a subunit mass of ~14 kDa. In human, the TTR subunit consists of 127 amino acid residues (Figure 1.9) (Kanda *et al*., 1974). Most of these amino acid residues are arranged into eight stranded β-sheets, and only 5% of them were found in a short α-helix portion (Figure 1.10 A) (Blake *et al*., 1974). Similarly to albumin but differ from thyroxine-binding globulin (TBG), TTR is not glycosylated. Heterogeneity of TTR observed in several animal species (Miller, 1963; Baker *et al*., 1966; Muto and Goodman, 1972; Taylor *et al*., 1975; Bhat *et al*., 1997; Harm *et al*., 1991). It was suggested as resulted from other modifications including dihydroxylation, phosphorylation, cysteine-glycine conjugation and glutathionylation (Terazaki *et al*., 1998). Moreover, heterogeneity of TTR resulted from complex formation of the protein with retinol binding protein (RBP) was also observed (Glover, 1973; Kopelman *et al*., 1976).

Figure 1.9   Amino acid sequence of human TTR

NH₂-Gly-Pro-Thr-Gly-Thr-Gly-Glu-Ser-Lys-Cys-Pro-Leu-Met-Val-Lys-Val-Leu-
Asp-Ala-Val-Arg-Gly-Ser-Pro-Ala-Ile-Asn-Val-Ala-Val-His-Val-Phe-Arg-Lys-
Ala-Ala-Asp-Asp-Thr-Trp-Glu-Pro-Phe-Ala-Ser-Gly-Lys-Thr-Ser-Glu-Ser-Gly-
Glu-Leu-His-Gly-Leu-Thr-Thr-Glx-Glx-Gln-Phe-Val-Glu-Gly-Ile-Tyr-Lys-Val-
Glu-Ile-Asp-Thr-Lys-Ser-Tyr-Trp-Lys-Ala-Leu-Gly-Ile-Ser-Pro-Phe-His-Glu-His-
Ala-Glu-Val-Val-Phe-Thr-Ala-Asn-Asp-Ser-Gly-Pro-Arg-Arg-Tyr-Thr-Ile-Ala-
Ala-Leu-Leu-Ser-Pro-Tyr-Ser-Tyr-Ser-Thr-Thr-Ala-Val-Val-Thr-Asn-Pro-Lys-
Glu-**COOH**

The three-dimentional structure of TTR purified from human serum has been determined by X-ray diffraction at both 2.5 Å (Blake and Oatley, 1977; Wojtczak, 1997) and 1.8 Å resolutions (Blake *et al*., 1978). It showed that about 45% of the amino acid residues of each TTR monomer are organized into eight β-sheets, which form into two four-strand β-sheets denoting as DAGH and CBEF. These eight strands of the β-sheet, then, form a β sandwich structure connected by the A-B loops (Blake *et al*., 1978). A short α-helix was found located at the end of strand E (Figure 1.10 B). The rest of amino acid residues were located in seven loops that connect the eight β-strands, segments of N-terminus (10 amino acid residues) and C-terminus (5 amino acid residues) (Hamilton *et a*l., 1993) located outside core structure of the tetramer. Two monomers of TTR joined edge-to-edge to form a dimer resulted in a pair of twisted eight-stranded of the β-sheets, an inner (DAGHH′G′A′D′) and an outer (CBEFF′E′B′C). The interactions between monomers involve hydrogen bonding between two F strands (F, F′) and two H strands (H, H′), however, H-strands are more extensively hydrogen bonded than the F strands. The tetrameric structure of TTR is formed from two dimers via hydrophobic interactions between amino acid residues in the A-B loop and the H strand of the opposite dimer (Figure 1.11). High content of β-structure (Hanlon *et al*., 1971; Branch *et al*., 1972), interaction between monomers to

form dimers and interaction between dimers to form tetramer contribute to an extraordinary stability of the TTR. In comparison, TTR in the tetrameric form is less stable than the dimer (Blake *et al.*, 1978).

A large central channel with approximately to 10 Å in diameter and 50 Å long and with sterically equivalent in thyroid hormone binding, but with differ in their relative binding affinities, is formed as a consequence of the tetrahedral arrangement of the TTR subunits (between DAGH) (Blake *et al.*, 1978). Amino acid residues that involve in ligand binding were located in strand A (residue 14 to 18), D (residue 53 to 56) and G and H (residue 105 to 122) (Neumann *et al.*, 2001). The chemical nature of the channel consists of three main elements. First, a hydrophilic center that forming from the hydroxyl groups of Ser and Thr residues, and associated bound-water molecules. Second, a hydrophobic patch that formed by the methyl group of Leu, Thr, Ala, Val and a group of charged residue such as Lys, Glu and His at which constitute around the entrance to the channel (for review see Blake, 1981; Klabunde *et al.*, 2000). And, finally, a negative cooperate binding of two molecules thyroid hormones with the amino acid residues inside the channel (Neumann *et al.*, 2001; Blake *et al.*, 1974). It revealed that only one binding site of TTR is occupied by thyroid hormone under physiological condition (Page *et al.*, 1973; Nilsson *et al.*, 1975).

The tetrameric form of TTR is stable and non-dissociated even in strong acidic or 0.1% sodium dodecyl sulfate (SDS) solution. But at some conditions such as at low pH (3.5 to 5.0) or high molar of denaturing agent, e.g. 4 M to 6 M guanidine hydrochloride (Guanidine-HCl), TTR tetramer were slowly dissociated into monomers (Kelly *et al.*, 1997; Lai *et al.*, 1997). Dissociation into dimers and incomplete dissociation into monomers of TTR tetramer were also demonstrated can occur in a solution of 5% SDS without heating and in 8 M urea, respectively (Rask *et al.*, 1971). In addition, the tetrameric dissociation into monomer of TTR was also suggested to occur at pH 7.0 and at nearly physiological ionic strength upon dilution to the sub molecular range (Quintas *et al.*, 1997; 1999). TTR lost the T4 binding activity when treated with 6 M urea, however, its binding activity could be restored when urea was removed out from the protein solution (Raz and Goodmann, 1969).
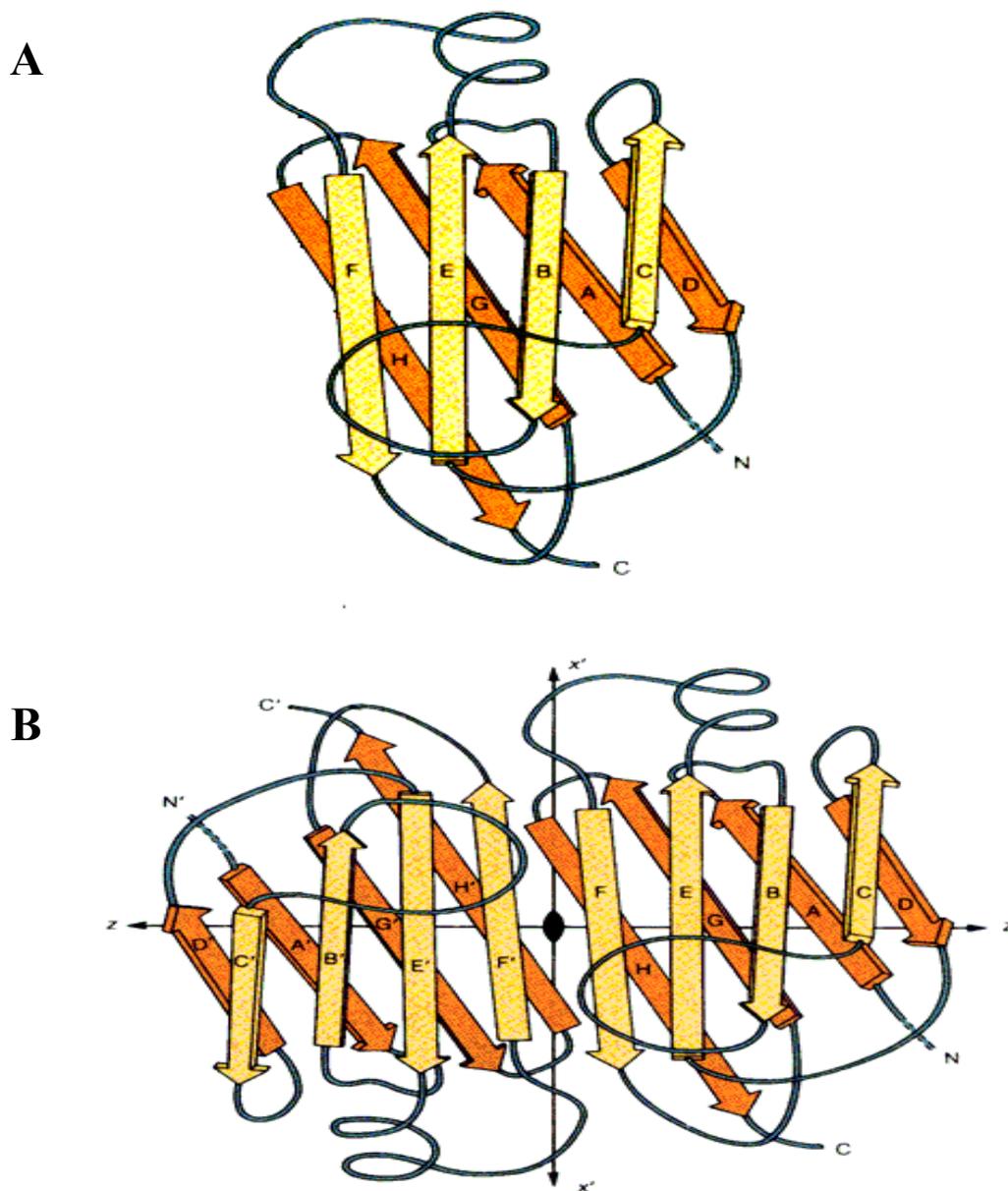
Figure 1.10   Structure of human TTR monomer (A) and dimer (B).

Each TTR monomer comprises two β-sheets those are formed from DAGH and CBEF strands, and one α-helix. Whereas, face-to-face interaction of the two β-sheets through a hydrogen bonding between HH' and FF' strands is the key for formation of dimer.

(Source: Blake *et al*., 1978)
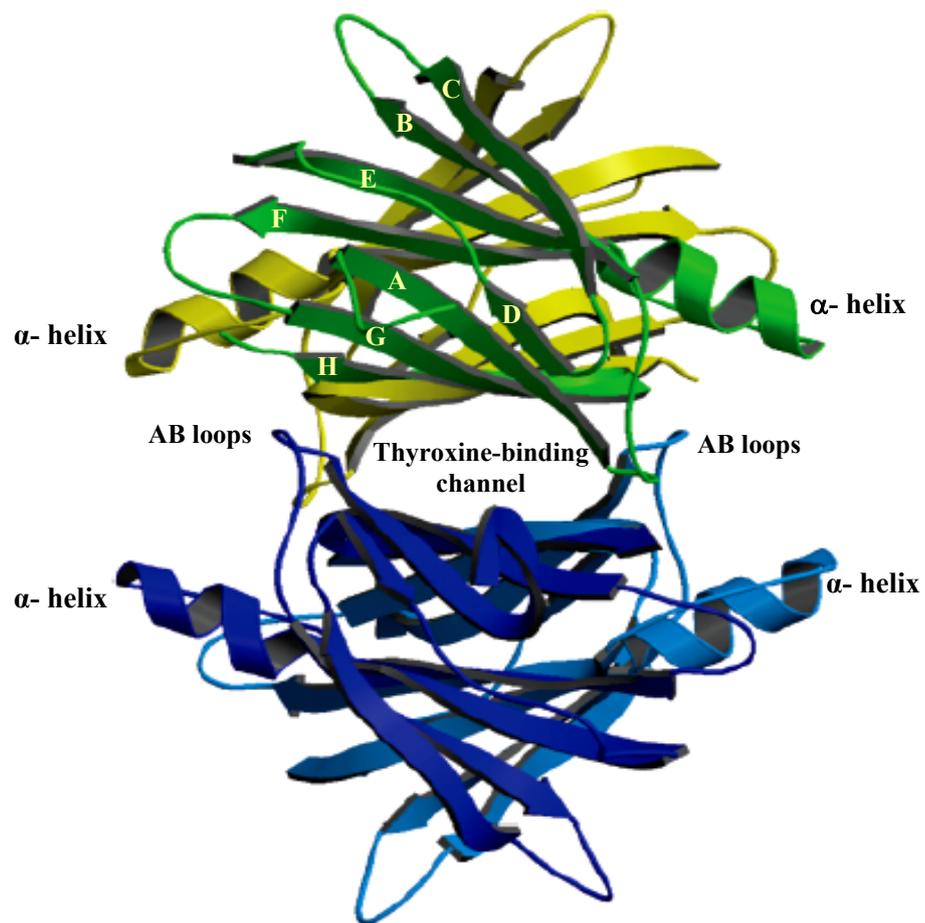
Figure 1.11 **The ribbon model of TTR Tetramer**

Two dimers of TTR subunits link together via AB loops to form a tetramer, and the thyroxin-binding channel was generated from the linking of the dimers.

(Source: Monaco, 2002)

2.3 Gene structure of human TTR

Each human TTR monomer is encoded by a single-copy gene (Sparkes et al., 1987) locating on the long arm of chromosome 18 (Wallace et al., 1985). It was found span to about 6.9 kilobases (kbp) containing four exons and three introns (Figure 1.12) (Sasaki et al., 1985). The nucleotide sequences at 5′ and 3′ flanking exon/intron border regions and introns of the TTR gene have been analyzed (Sasaki et al., 1985; Fung et al., 1988; Motojima and Goto., 1990). It showed that nucleotide sequence of the TTR exon 1 was 95 base pairs (bp), encoding for twenty amino acid residues of a signal peptide and three amino acid residues of the mature protein. Whereas, exon 2, exon 3 and exon 4 contain 131, 136 and 254 bp encoding for 44, 45 and 35 amino acid residues, respectively. The distance between the transcriptional initiate site and the polyadenylate (poly (A)) site is 6,931 bp. The sequence of poly (A) tail is located 123 bp downstream of the coding region (Sasaki et al., 1985). Lengths of the three introns are 934, 2090 and 3308 bp, respectively. The consensus sequences for splicing, GT and AG, were found at the boundaries of all three introns (Breathnatch et al., 1978). Two sequence copies of Alu family were found in intron 2 and 3 of the TTR gene, and it was suggested to involve in the gene expression and regulation. The repeated sequences at 3′ untranslated region were found almost identical to the core sequences. However, biological function of these repeated sequences is still unknown. The sequence of TTR gene is highly conserved through evolution. More than 80% identity in the sequence was reported among the mammalian TTRs (Wakasugi et al., 1985; Schreiber and Richardson, 1997).
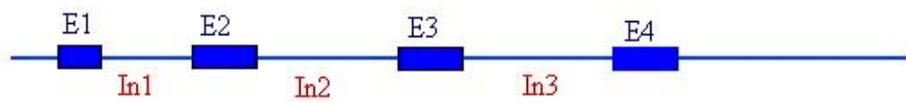
Figure 1.12   Diagram of TTR gene structure

Close boxes with E1, E2, E3 and E4 on top represent TTR exon 1, 2, 3 and 4, respectively. Blue line with In1, In2 and In3 underneath represent TTR intron1, 2 and 3, respectively.

2.4   Synthesis and distribution of TTR

TTR is mainly synthesized in liver (Felding and Fex, 1982) and epithelial cells of choroid plexus (Aleshire *et al.*, 1983). In addition, it is also found synthesized in other cells including the retinol pigment epithelial cells (Martone *et al.*, 1988; Herbert *et al.*, 1991; Ong *et al.*, 1994). TTR in blood circulation is mainly produced in hepatocytes, and then secreted into the blood (Dickson *et al.*, 1986). It is synthesized as a pre-protein with a signal peptide at N-terminus, which is cleaved during translocation of the protein into an endoplasmic reticulum (ER), and within the ER of hepatocytes, TTR tetramer is formed (Bellovino *et al.*, 1996; 1998). TTR is distributed throughout the body and in equilibrium with the interstitial fluid by penetrating the capillaries. The plasma level of TTR increases successively after birth and reaches 250µg/ml when adult (Smith and Goodman, 1971). However, the concentration of plasma TTR decreases after the fifth decade of life.

TTR in liver is one of a negative acute phase reactant. The rate of TTR synthesis decreases during acute phase response to trauma and chronic inflammation (Birch and Schreiber, 1986; Milland *et al.*, 1990; Richardson *et al.*, 1998). Low concentration of TTR may result in higher concentration of free ligand, e.g. thyroxine and retinol, which lead to malnutrition. In addition, it can reflect the metabolic competence of body (for reviews see Raghu and Sivakumar, 2004). Although, synthesis of TTR by liver may be altered under stress condition, the synthesis of TTR in choroid plexus was very slightly affected (Dickson *et al.*, 1982). This indicated that the synthesis of TTR in liver and choroid plexus is independently regulated during the acute phase response (Dickson *et al.*, 1986).

Choroid plexus, an epithelial membrane component of blood-brain barrier, is the main synthesis site of TTR found in CSF (Cserr, 1971). All of TTR synthesis by the choroid plexus is secreted towards the brain and has been proposed to be involved in transport of T4, but not T3, from blood to brain (Dickson *et al.*, 1987). Localization of TTR in choroid plexus was first demonstrated using an immunohistochemical technique (Aleshine *et al.*, 1983), and it was found distributed in cytoplasm and epithelial cell of the choroid plexus (Herbert *et al.*, 1986). TTR mRNA was located in the epithelial cells prior to being secreted into the CSF (Southwell *et al.*, 1993). Its concentration in choroid plexus was much higher than that

in liver (Schreiber *et al.*, 1990). About 50% of total secreted protein and about 12% of the synthesized protein in choroid plexus is TTR (Dickson *et al.*, 1986). In mammal and avian, the TTR gene was expressed in the cells of the choroid plexus in early stage of embryogenesis before the formation of blood brain-barrier (Southwell *et al.*, 1991) and during fetal development (Thomas *et al.*, 1988). A correlation between growth rate of the brain and expression level of TTR gene was evidence in growing animal (Southwell *et al.*, 1991).

2.5 Functions of TTR

The most well-known physiological function of TTR is a protein transporter for thyroid hormones and vitamin A. Thyroid hormones, both T4 and T3, directly bind to TTR whereas vitamin A (in retinol form) is bound to TTR through the mediation of retinol-binding protein (RBP).

2.5.1 as a thyroid hormone distributor protein

TTR is the one of thyroid hormones binding protein in plasma of higher vertebrates, besides thyroid hormone binding globulin (TBG) and albumin. In human, binding affinity of TTR to thyroid hormones, in particular T4, is less than TBG but higher than albumin (for review sees Schreiber and Richardson, 1997). About 10-15% (Palha, 2002) and up to 80% (Hagen and Elliot, 1973) of T4 in human plasma and in central nervous system, respectively, are bound and transported by TTR. Human TTR has higher affinity for T4 than T3, i.e. T4 bound to TTR about 10 times higher than T3. However, this binding affinity was change during evolution of vertebrates (for review see Schreiber and Richardson, 1997; Schreiber *et al.*, 1998)

TTR has two binding sites for thyroid hormones (Blake *et al.*, 1978). At the physiological condition, only one site is occupied by the hormone molecule due to the negative cooperativity of thyroid hormone binding. By equilibrium dialysis measurement, the association constant (Ka) was 1.0×10^8 M^{-1} and 9.5×10^5 M^{-1} for the first and second ligand binding sites, respectively (Ferguson *et al.*, 1975), suggesting that binding of the first ligand was much stronger than the second ligand. This cooperative effect is involved in the conformational changes induced by ligand binding to TTR molecule (Neumann *et al.*, 2001). By comparing the TTR channel

diameter of the two binding sites, it revealed that binding of the hormone to the first site can change diameter of the second. The outer and inner pockets of the channel (strand A, D and H) were wider while the middle part (strand G) was collapsed (Neumann *et al.*, 2001). Therefore, the second hormone molecule bound to the binding site with less strength.

The tissue distribution of TTR synthesis was observed by intravenous injection of ^{125}I-labeled T4 to various tissues (Dickson *et al.*, 1987). T4 was found first strongly accumulation in the choroid plexus. Then, it was transported across the blood brain barrier into CSF by binding with TTR (Southwell *et al.*, 1993). This revealed the hypothetical mechanisms for T4 transport from blood to brain in such a way that T4 enter to the brain by moving across the epithelial cells of choroid plexus or moving across the cerebral endothelial cells. In moving across, T4 bound to TTR that locates within the choroid plexus or within CSF (Figure 1.13.). In addition, as the concentration of TTR in CSF was related to the total concentration of T4 that transported to the brain and between tissue and extracellular fluid, the mechanism of regulation of T4 level in the brain was proposed.

2.5.2 as a carrier protein for retinol via binding to RBP

TTR also binds and transports vitamin A or retinol through the binding to RBP, a vitamin A carrier protein that is a 21-kDa single polypeptide chain protein found in plasma of vertebrates. The TTR-RBP complex transports retinol from the main storage in liver to target cells in peripheral tissues. It was shown that without binding to TTR, RBP can be eliminated by glomerular filtration in kidney because of its small molecule. Therefore, the binding of RBP to TTR was thought to prevent the loss of RBP and retinol through glomerular filtration in kidney (Kanai *et al.*, 1968; Goodman and Raz, 1972). In human plasma, about 40% of TTR circulates as the complex with RBP (Smith and Goodman, 1971), while less than 1% circulates as the complex with T4 (Woeber and Ingbar, 1968). The complex formation of TTR and RBP was found occurred at physiological pH and involved with a hydrophobic interaction (Peterson, 1971). The complex dissociated readily at low ionic strength and in the presence of 6 M urea.
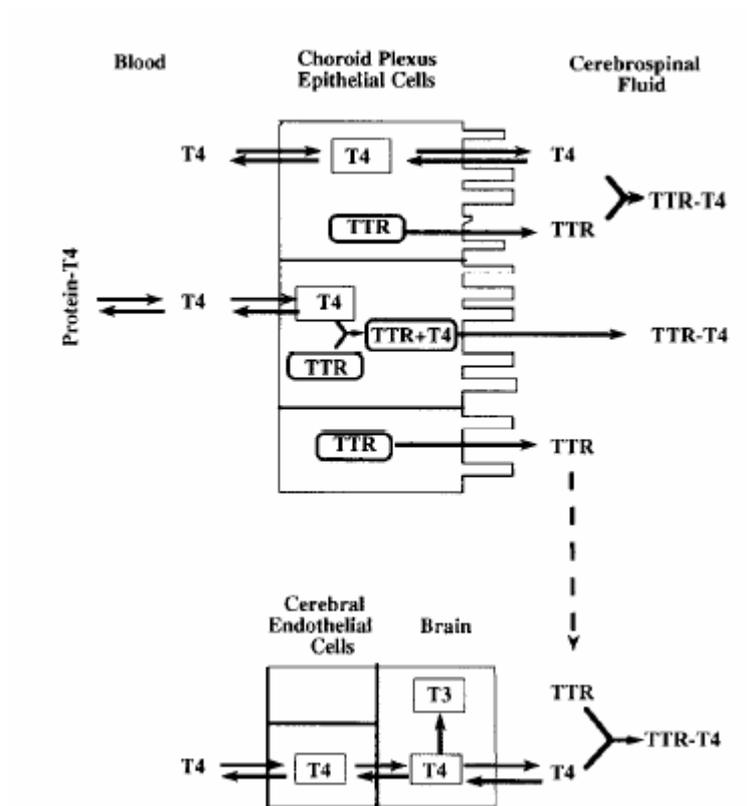
Figure 1.13 **The hypothetical mechanisms for the role of TTR within choroid plexus in moving T4 from the blood to brain**

The diagram shows the hypothetical routes for T4 movement from blood to brain and CSF. T4 may enter the brain by moving across the choroid plexus epithelial cell (the upper) or moving across the cerebral endothelial cells (the lower). T4 does not undergo deiodination in the choroid plexus but conversion of T4 to T3 is occurred within the brain tissue by deiodinases.

(Sources: Southwell *et al.,* 1993; Schreiber and Richardson, 1997)

In human plasma, TTR and RBP formed complex with 1:1 molar ratio though the *in vitro* studying suggested more than one binding sites for RBP (one to four sites) are on a TTR molecule (Raghu *et al.*, 2003). This was probably due to the limit concentration of RBP in comparing to that of TTR. The binding sites for T4 and RBP are independent from each other (Eneqvist *et al.*, 2001). This evidenced from that binding of RBP did not change the binding capacity of TTR to T4 (Nilsson and Peterson, 1971).

2.5.3 Other functions

A thymic hormone-like activity influence of TTR was demonstrated in spleen cells of thymectomized rats (Burton *et al.*, 1978, 1985). It indicated that TTR possessed immuno-potentiating properties *in vitro* and *in vivo* (Burton *et al.*, 1985). Induction with TTR enhanced synthesis of IgM, and a decrease in the specific activity of thymocyte terminal deoxynucleotidyl transferase was also observed. Moreover, the amino-terminal domain of the TTR molecule was shown containing a property similar to T-lymphocytes (Burton *et al.*, 1987).

TTR has been shown interacting with perlecan, an important constituent of basement membranes and connective tissues (Iozzo *et al.*, 1994) and has been identified as a common proteoglycan componebt occurring in several types of amyloidosis (Kisilevsky *et al.*, 1992; Magnus *et al.*, 1992). It has been proposed that the binding of perlecan to TTR depends on hydrophobic interactions between the two proteins. Binding of perlecan to TTR resulted in sequestering the protein from the retinol-RBP-TTR complex (Smeland *et al.*, 1997), thus affecting metabolism of retinol. Moreover, interaction between TTR and perlecan was suggested favor with the co-localization of these two proteins in the TTR-associated amyloidotic deposits (Smeland *et al.*, 1997).

Recently, TTR has been reported having the proteolytic activity (Liz *et al.*, 2004). It was shown that TTR can proteolytical process the C-terminal of apolipoprotein A-1, thus, was suggested may interact with other substrates *in vivo* under physiological and pathological condition.

3. Amyloidosis

Amyloidogenic TTR mutation

Hereditary TTR amyloidosis is a genetically transmitted disease that resulted from mutation of TTR gene (Saraiva *et al*., 1984). The mutation leads to the extracellular deposition of the TTR subunits in tissue as amyloid. The deposition of TTR amyloid in peripheral nerve and the heart associated with neuropathy and cardiomyopathy, respectively. The most of familial amyloidotic polyneuropathy (FAP) are related to the TTR gene point mutation occurring in adult. The most frequent variant that incident around the world is Val30Met (V30M; substitution of valine by methionine at amino acid position 30). This TTR variant is resulted from single mutation of the nucleotide sequence in exon2 in which guanine is substituted by adenine (Saraiva *et al*., 1984). This TTR genetic abnormality presents in Portuguese, Swedish, Japanese, Majorca and French (Saraiva *et al*., 1984; Andersson, 1970; Araki, 1984; Munar-Ques *et al*., 1997; Planté-Bordeneuve *et al*; 1998). The clinical symptom of FAP began in the third to forth decade of life with early impairment of temperature, pain sensation in feet, autonomic dysfunction, electrocardiographic abnormally emaciation and death finally (Andrade, 1952). A few of TTR mutations found related to cardiomyopathy without neurological symptom. Among of these, Val122Ile (V112I; substitution of valine by isoleucine at amino acid position 112) is the most frequent distributed in African Americans (Jacobson *et al*., 1997). Until now, over 100 TTR variants were reported and 80 of them are pathogenic and related to amyloidosis (Saraiva, 1996).

Amylodoisis is a group of diseases in which proteins or protein fragments change from their native soluble forms into an insoluble fibril called "amyloid". The amyloid fibrils have been found accumulated in a variety of organs and tissues of the body including peripheral nerves, kidney, gastrointestinal tract, heart and thyroid (Kelly, 1996; Rochet and Lansbury, 2000). The accumulation of large amounts of amyloid fibrils can result in damaging of function and structure of organs. Up to date, 24 different amyloidogenic proteins have been identified in human, and they are associated with several disorders including AD (Selkoe, 1996), Parkinson's

disease (Conway *et al.*, 1998), familial amyloidotic polyneuropathy (FAP) (Costa *et al.*, 1978; Mc Cutchen *et al.*, 1995), light chain amyloidosis (Buxbaum and Gallo, 1999), and dialysis-related amyloidosis (Drueke, 2000).

The "amyloid" was first used to describe the cerebral corpora amylacea, a material that was found during histopathological examination of brain (Virchow, 1854), which was later identified as a carbohydrate substance. In 1859, it was shown that protein was a component of amyloid (Friedreich and Kelkulé, 1859). By detection using Congo red, an amorphous proteinaceous aggregates were identified (Bennhold, 1922). The fibrillar ultrastructure of the amyloid fibrils was later revealed by electron microscopy (Cohen and Calkins, 1959).

3.1 Nature of amyloid

Amyloid is an insoluble fibrillar protein that extracellularly deposit in tissues or organs. The amyloid fibrils can be formed from several different precursor proteins that share a common ultrastructure. These proteins exhibit as straight and non-branch fibrils with about 7.5 to 10 nm thick but undetermined length, and reveal a superficial twist and two protofibrils (Cohen, 1966). The X-ray diffraction showed that the fibril has a "cross β" structure in which the constituent strands are arranged perpendicular to the long axis of the fibril (Blake and Serpell, 1996). Moreover, the amyloid fibril showed uniform tintorial properties including apple green birefringence after staining with Congo red (Glenner, 1981; Serpell *et al.*, 1997). The amyloid fibrils showed orange colored fluorescence that is most sensitive (Linke, 2000).

Many mechanisms have been proposed for the conformational change of the precursor protein that leads to the fibril formation. These include point mutation (Saraiva, 2001; Shastry, 2003), overproduction of the precursor protein (Gillmore *et al.*, 2001), change in local pH of membrane, oxidation and proteolytic cleavage (Haass and Steiner, 2001). However, several amyloid fibrils have been demonstrated forming from the wild type of precursor proteins (Westermark *et al.*, 1986). The precursor proteins that adopt a globular folding in their native state require partial unfolding of their tertiary structure prior to the fibril formation, while those unfolded

in their native state required a transition to an ordered secondary structure (Rochet and Lansbury, 2000).

3.2 Classification of amyloid

The amyloid disease can be divided into two groups according to their distribution in body, the localized and the systemic amyloidoses. For the localized amyloids, the deposition is restricted to one tissue or organ whereas the systemic amyloids deposit in many different organs or tissues. All amyloid precursor proteins have been identified so far are diverse and unrelated, but produce amyloid deposition with beta-fibrillar as a common structure. The specific precursor proteins and their classification are listed in Table 1.1

3.3 TTR amyloidosis

TTR is one of the precursor proteins known to form amyloid fibril (Damas and Saraiva, 2000) that is associated with systemic amyloidosis. The TTR amyloidosis, including familial amyloidotic polyneuropathy (FAP) and cardiomyopathy (FAC), are caused by mutation of the TTR gene. Up to date, more than 80 point mutations of TTR have been identified and most of them were revealed associated with FAP. Only about 12 of the mutations are nonpathogenic (Connors *et al.*, 2003; Saraiva, 2001). The mutate TTRs have been reported to be the non-causative molecules for the senile systemic amyloidosis (SSA). However, this disease is a sporadic disorder resulted from deposition of the wild-type TTR fibril in cardiac and other tissues.

Table 1.1   **Human amyloid fibril proteins and their precursors (Source :**
Westermark *et al*., 2002)

Amyloid protein	Precursor	Systemic (S) or Localized (L)	Syndrome or tissue of deposition
AL	Immunoglobulin light chain	S, L	Primary, Myeloma associated
AH	Immunoglobulin heavy chain	S, L	Primary, Myeloma associated
ATTR	Transthyretin	S	Familial, Senile systemic
Aß$_2$M	β_2-microglobulin	S	Hemodialysis
AA	(Apo)serum AA	S	Secondary, reactive
AApoA-I	Apolipoprotein A-I	S	Familial
AApoA-II	Apolipoprotein A-II	S	Familial
AGel	Gelsolin	S	Familial
ALys	Lysozyme	S	Familial
AFib	Fibrinogen α-chain	S	Familial
ACys	Cystatin C	S	Familial
ABri	ABriPP	L	Familial dementia
ADan	ADanPP	L	Familial dementia
Aß	Aβ protein precursor (AβPP)	L	Alzheimer's disease, aging
APrP	Prion protein	L	Spongioform encephalopathies
ACal	(Pro)calcitonin	L	C-cell thyroid tumors
AIAPP	Islet amyloid	L	Islets of Langerhans
AANF	polypeptide	L	insulinomas
APro	Atrial natriuretic factor	L	Cardiac atrial,
AIns	Prolactin	L	Aging pituitary, Prolactinomas
AMed	Insulin	L	Iatrogenic
Aker	Lactedherin	L	Senile aortic, media
A(tbn)	Kerto-epitelin	L	Cornea; Familial
ALac	To be named Lactoferrin	L	Pindborg tumors Cornea; Familial

3.3.1 Familial amyloidotic polyneuropathy (FAP)

FAP is the most common hereditary systemic amyloidosis. It is identified as an autosomal dominant disorder with peripheral sensory motor and autonomic neuropathy (Andrade, 1952). It is well known that the amyloidogenic TTR is a major constituent of the amyloid that deposits in tissue of the FAP patients. The disorder of FAP was first observed in the Portuguese (Andrade, 1952). The amyloid fibrils were shown extracellularly systemic deposited throughout the connective tissue with the exception of brain and liver parenchyma. It affected particularly the peripheral nervous system (Coimbra and Andrade, 1971a, b) and led to dysfunction of organs and ultimately death. The symptom onset of FAP usually occurs in the third to the forth decade of life. The symptom usually begins with a neuropathy of the sensory peripheral in lower limbs following by the motor impairments, which leads to wasting and weakness (Andrade, 1952; Booth *et al.*, 1998; Misrahi *et al.*, 1998). Most of FAP patients involve with an early and severe autonomic nervous system, commonly manifested by dyshydrosis, sexual impotence, alternating diarrhea and constipation as well as orthostatic hypotension and urinary bladder dysfunction (Canijo and Andrade, 1969; Guimarães *et al.*, 1980; Alves *et al.*, 1997b; Ando and Suhr, 1998).

The biochemical nature of amyloid deposits was elucidated. It showed mutated TTR as the main protein constituent of the amyloid deposits in FAP (Costa *et al.*, 1978). Amongst, TTR V30M is the most common and has been found throughout the world, however, in endemic areas. It is frequently found in northern of Sweden (Andersson, 1976; Holmgren *et al.*, 1994), northern of Portugal (Andrade, 1952; Alves *et al.*, 1997a), and Japan (Araki *et al.*, 1968; Araki, 1984). Prevalence and age of the disease onset vary among and within populations. In Portugal, prevalence of V30M is high and the symptoms typically develop before the age of 40 years (Sousa *et al.*, 1995). A slightly lower prevalence, to approximate 5%, and later onset, usually after the age of 50 years, of the disease was reported in Swedish carriers (Andersson, 1976; Sousa *et al.*, 1993). Onset of the disease reported in the Japanese was either early or late (Tashima *et al.*, 1995). Incidentally, the V30M homozygous did not show more severe form of FAP or some did not, even, develop FAP (Holmgren *et al.*, 1988; Yoshinaga *et al.*, 1992), indicating that mutation itself is

necessary but not sufficient to cause FAP. Other unidentified genetic or environmental factors were suggested contribute to the pathogenesis of the disease.

Up to date, more than 80 mutations of TTR gene have been reported in association with human amyloidosis (see Table 1.2) (Saraiva, 2001), and most of them are connected to FAP with an indistinguishable clinical symptom from the original description of the disease (Toyooka *et al.*, 1995; Booth *et al.*, 1998; Misrahi *et al.*, 1998; de Carvalho *et al.*, 2000). Whereas, the others may contribute to a variety of phenotype including simultaneous neuropathy and cardiomyopathy, carpal tunnel syndrome, predominance of vitreous TTR deposition, and involvement of leptomeningeal. Only a few TTR mutations are related to cardiomyopathy without neurological symptoms. The most common TTR mutation associated with familial cardiac amyloidosis (FAC) is V122I, frequently found in the African-Americans. The allele frequency of V122I among the population was about 12% (Jacobson *et al.*, 1997)

Among the TTR mutations, some have been reported to appear as non-amyloidogenic (see Table 1.3). Frequency of each allele is varied in different populations. For examples, Gly6Ser (G6S; substitution of glycine by serine at amino acid position 6) is present about 12% of the Caucasian population, while, Thr119Met (T119M; substitution of threonine by methionine at amino acid position 119) is found to about 0.8% of Portuguese and German populations. However, in some cases where both pathogenic and non-pathogenic mutations occurred, the non-pathogenic mutation apparently inhibited the development of FAP (Coelho *et al.*, 1996)

Table 1.2   TTR amyloidogenic variants (Source: Saraiva., 2001)

Muatation	Codon change		Predominant clinical feature	Origin
Cys10Arg	TGT	CGT	PN, AN, Eye	Hungary
Leu 12Pro	CTG	CCG	LM, PN, AN	UK
Asp18Glu	GAT	GAG	PN, AN	Columbia
Asp18Gly	GAT	GGT	LM	Hungary
Val20Ile	GTC	ATC	Heart	Germany
Ser23Asn	AGT	AAT	Heart	Portugal
Pro24Ser	CCT	TCT	Heart, CTS, PN	USA
Val28Met	GTG	ATG	PN, AN	Portugal
Val30Met	GTG	ATG	PN, AN, Eye	Several
Val30Ala	GTG	GCG	Heart, AN	Germany
Val30Leu	GTG	CTG	PN, AN	Japan
Val30Gly	GTG	GGG	LM, Eye	France
Phe33Ile	TTC	ATC	PN, Eye	Poland
Phe33Leu	TTC	CTC	PN, AN	Poland
Phe33Val	TTC	GTC	PN, AN	UK
Arg34Thr	AGA	ACA	PN, Heart	Italy
Lys35Asn	AAG	AAC	PN, AN, Heart	France
Ala36Pro	GCT	CCT	PN, Eye	Greece
Asp38Ala	GAT	GCT	PN, Heart	Japan
Glu42Gly	GAG	GGG	PN, AN	Japan
Glu42Asp	GAG	GAT	Heart	France
Phe44Ser	TTT	TCT	PN, AN, Heart	Ireland
Ala 45Asp	GCC	GAC	Heart	Italy
Ala45Ser	GCC	UCC	Heart	Sweden
Ala45Thr	GCC	ACC	Heart	Italy
Gly47Arg	GGG	CGG	PN, AN	Japan
Gly47Ala	GGG	GCG	Heart, PN, AN	Italy

Table 1.2 (continued)

Mutation	Codon change		Predominant clinical feature	Origin
Gly47Glu	GGG	GAG	PN	Germany
Thr49Ala	ACC	GCC	Heart, PN	Italy
Thr49Ile	ACC	ATC	PN, Heart	Japan
Ser50Arg	AGT	AGG	PN, AN	Japan
Ser50Ile	AGT	ATT	Heart, PN, AN	Japan
Glu51Gly	GAG	GGG	Heart	USA
Ser52Pro	TCT	CCT	PN, AN, Heart	UK
Gly53Glu	GGA	GAA	LM, Heart	France
Glu54Gly	GAG	GGG	PN, AN	UK
Glu54Lys	GAG	GAA	PN, AN, Heart	Japan
Leu55Arg	CTG	CGG	LM, PN	Germany
Leu55Pro	CTG	CCG	PN, Heart, AN	Taiwan
His56Arg	CAT	CGT	Heart	USA
Leu58His	CTC	CAC	CTS, Heart	Germany
Leu58Arg	CTC	CGC	CTS, AN, Eye	Japan
Thr59Lys	ACA	AAA	Heart, PN	Italy
Thr60Ala	ACT	GCT	Heart, CTS	Ireland
Glu61Lys	GAG	AAG	PN	Japan
Phe64Leu	TTT	CTT	PN, CTS, Heart	Italy
Phe64Ser	TTT	TCT	LM, PN, Eye	Italy
Ile68Leu	ATA	TTA	Heart	Germany
Tyr69His	TAC	CAC	Eye	Scotland
Lys70Asn	AAA	AAC	CTS, PN, Eye	Germany
Val71Ala	GTG	GCG	PN, Eye	Spain
Ile73Val	ATA	GTA	PN, AN	Bangladesh
Ser77Phe	TCT	TTT	PN	France
Ser77Tyr	TCT	TAT	PN	Germany

Table 1.2 (continued)

Mutation	Codon change		Predominant clinical feature	Origin
Ile84Asn	ATC	AAC	Eye, Heart	Italy
Ile84Thr	ATC	ACC	Heart, PN, AN	Germany
Glu89Gln	GAG	CAG	PN, Heart	Italy
Gku89Lys	GAG	AAG	PN, Heart	USA
Ala91Ser	GCA	TCA	PN, CTS, Heart	France
Ala97Gly	GCC	GGC	Heart, PN	Japan
Ala97Ser	GCC	TCC	PN, Heart	France
Ile107Val	ATT	GTT	Heart, CTS, PN	Germany
Ile107Met	ATT	ATG	PN, Heart	Germany
Ala109Ser	GCC	TCC	PN	Japan
Leu111Met	CTG	ATG	Heart	Denmark
Ser112Ile	AGC	ATC	PN, Heart	Italy
Tyr114Cys	TAC	TGC	PN, AN, Eye	Japan
Tyr114His	TAC	CAC	CTS	Japan
Tyr116Ser	TAT	TCT	PN, CTS	France
Ala120Ser	GCT	TCT	Heart, PN, AN	Africa
Val122Ile	GTC	ATC	Heart	Africa
Val122del	GTC	Loss	Heart, PN, CTS	Equador/Spain
Val122Ala	GTC	GCC	Heart, Eye, PN	UK

AN, autonomic neuropathy; CTS, carpal tunnel syndrome; Eye, vitreous deposition; PN, peripheral neuropathy; LM, leptomenigeal amyloid; Heart; cardiomyopathy.

Table 1.3 TTR non-amyloidogenic variants

Gly6Ser, Met13Ile, Asp74His, His90Ans, Gly101Ser, Pro102Arg, Arg104Cys, Arg104His, Ala109Thr, Ala109Val, Thr119Met, Pro125Ser

3.3.2 Senile systemic amyloidosis (SSA)

Senile systemic amyloidosis (SSA) is the most common amyloidosis caused by the deposition of wild-type TTR (wtTTR) fibrils. It is the most prevalent systemic form of amyloidosis, at least in the western. Screening of Swedish and American above 80 years of age revealed that 25% to 28% of the people showed some degree of the disease (Westermark *et al.*, 1979; Cornwell *et al.*, 1983; Westermark *et al.*, 1990). However, it was rarely seen in individuals younger than 70 years (Röcken *et al.*, 1994). Pathogenesis of the TTR amyloid deposits are commonly found not only in heart, but also in lungs, blood vessels and the renal medulla of kidneys (Westermark *et al.*, 2003). SSA is typically manifested by cardiac disorders with congestive heart failure, arrhythmia and conduction blocks, and sometimes carpal tunnel syndrome.

The pathogenesis of SSA is unknown. There is no mutation (Westermark *et al.*, 1990; Gustavsson *et al.*, 1995) and no evidence of over-expression of the TTR gene. On the other hand, the level of TTR in plasma slightly decreased in SSA (Westermark *et al.*, 1985). Most of the TTR molecules found in the fibril of SSA are fragmented, leading to a postulation on an important role of fragmentation in pathogenesis of the SSA (Felding *et al.*, 1985; Westermark *et al.*, 1990; Westermark *et al.*, 2003). The fragmentation of TTR molecule is not a random process because all of cleaved peptide bonds are situated within the C-strand, C-D loop and D-strand of the TTR polypeptide. However, it is not known whether the proteolytic cleavage plays a role in formation of TTR fibrils or merely represents a phenomenon of the post-amyloidogenic. Some post-translational modifications of wtTTR such as S-sulfonation and thiol-conjugation of the cysteine residue at position 10 of the TTR polypeptide

have been implied to increase amyloidogensesis (Kishikawa *et al.*, 1999; Suhr *et al.*, 1999).

3.4 Fibrillogenesis of TTR

Several different forms of TTR have been suggested as precursors of the TTR amyloids. These include monomer, whole dimer, truncated dimer and tetramer forms (Lai *et al.*, 1996; Lashuel *et al.*, 1998; Schormann *et al.*, 1998; Quintas *et al.*, 1999; Eneqvist *et al.*, 2000; Quintas *et al.*, 2001; Serag *et al.*, 2001). Several models of TTR amyloid formation have been proposed. However, in general, all of them were originated primarily on the formation that begins with the disruption of the protein tetramer into monomers (Figure 1.14). Tetramer has been believed separating into AB and CD dimers that further rapidly dissociate into monomers. Neither dissociation into AC- and BD-dimers nor sequential dissociation into monomers was suggested. These monomers are, then, undergo a conformational change, which promotes the formation of the amyloid fibrils (Figure 1.14) (McCutchen and Kelly, 1993; Lai *et al.*, 1996; Kelly, 1998; Nettleton *et al.*, 1998; Quintas *et al.*, 1999; Jiang *et al.*, 2001; Wiseman *et al.*, 2005; Foss *et al.*, 2005).

Three dimensional studies by X-ray crystallography of TTR structure suggested that several mutations of TTR polypeptide would destabilize the native tertiary structure of the protein. The largest change in the tertiary structure was observed in Leu55Pro (L55P; substitution of leucine by proline at amino acid position 55). This finding is related to the clinical observation showing high tendency in amyloidogenic fibrillar forming of L55P (Sebastião *et al.*, 1998). It was also shown that the mutations are predominantly situated in the hydrophobic core of TTR. The mutation was usually concentrated within C-strand, C-D loop and D-strand of each TTR monomer (Serpell *et al.*, 1996; Eneqvist and Sauer-Eriksson., 2001), and has been suggested involving in the structural changes required for amyloid formation (Lai *et al.*, 1996). Not all of TTR mutations lead to the disease. Some mutations were reported increase stability of the tetrameric structure of TTR. The *in vitro* studies on double mutations such as V30M/T119M showed that these variants exhibited stability of the tetramer very close to that of the wtTTR (Almeida *et al.*, 2000).
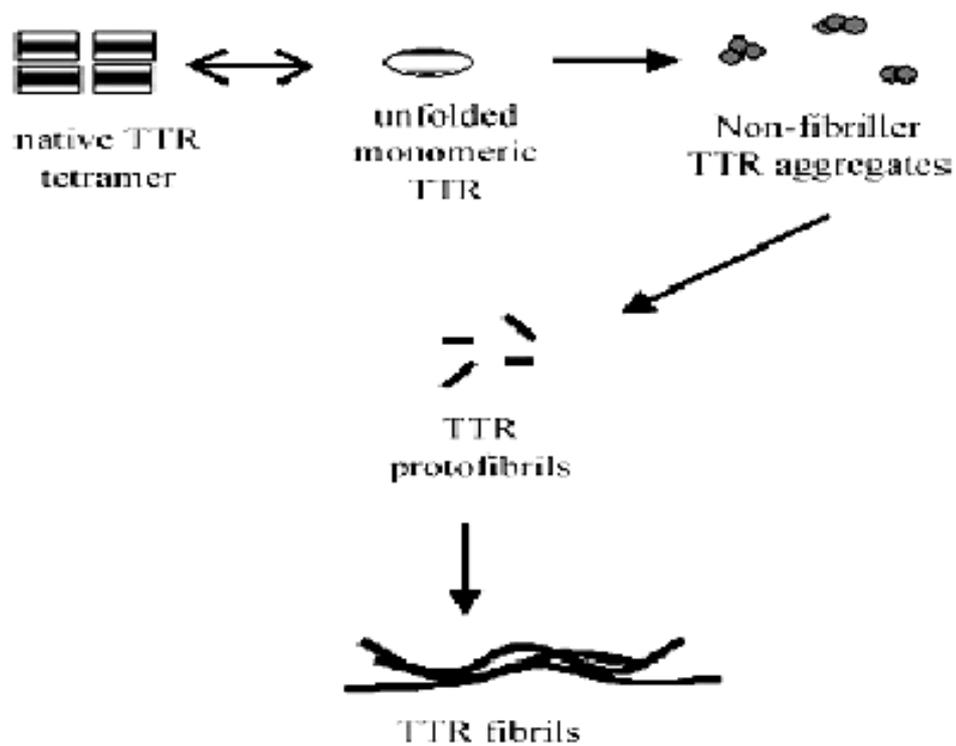
Figure 1.14   The proposed pathway of TTR fibril formation

Native TTR tetramer dissociates into unfolded TTR monomers, which self-aggregated originating non-fibrillar TTR aggregates, protofibrils and mature amyloid fibrils.

(Source: Sousa, 2003)

Although liver transplantation seems to be the only capable method of preventing progression of the disease, considerable risks for the patients and only slight improvements in neurological function are the limits (Lewis *et al.*, 1994). Less invasive therapies that target to reduce amount of amyloid in organs such as preventing the dissociation of TTR tetramer into monomers and disrupting the amyloid aggregates is being pursued (Figure 1.15). Several small molecules such as tetracycline and T4 that show ability in binding to the thyroid hormone binding channel of TTR have been demonstrated to stabilize the TTR tetramer and inhibit dissociation into monomer species (Miroy *et al.*, 1996; Peterson *et al.*, 1998; Almeida *et al.*, 2004). These molecules were demonstrated to inhibit oligomers of both wtTTR and TTR variants such as V30M and L55P *in vitro* (Miroy *et al.*, 1996; Peterson *et al.*, 1998; Almeida *et al.*, 2004).
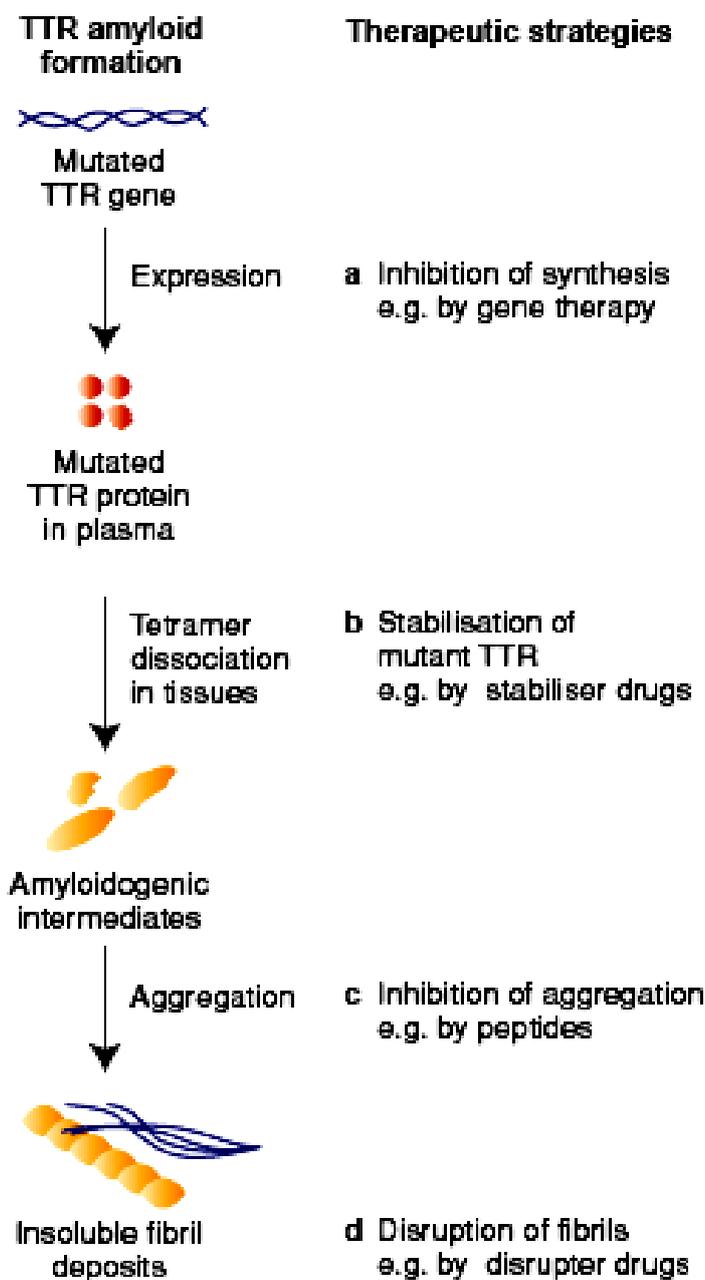
Figure 1.15   Potential molecular therapies for the TTR amyloidoses.

Procedures that might block the amyloidogenicity of TTR include (a) inhibition of synthesis of mutated TTR, (b) stabilization of the soluble circulating amyloid precursor, (c) inhibition of aggregation of the amyloidogenic intermediates and (d) disruption of the insoluble deposits.

(Source : Saraiva, 2002)

Objectives

1. Screening for ApoE genotype and allele variant in Thai people
2. Screening for and characterize TTR variant in Thai people

CHAPTER 2

MATERIAL AND METHOD

1. Materials

1.1 Instruments

Instrument	Model	Company
Autoclave	ES-315	Tomy
Automat DNA sequencer	ABI PRISM 377	Applied Biosystem
Balance (4 digit)	AB204-S	Mettler
Balance (2 digit)	PG5002-S	Mettler
Centrifuge	5804R	Eppendorf
Centrifuge	18/80(MSE)	Harrier
Gel Document (Labworks 4.0)	C-80	UVP
Gel Document	Biodoc-It™ System	UVP
Horizontal Electrophoresis	B1	Owl Scientific
Incubator		Memmert
Microcentrifuge	260D	DENVILLE
Microcentrifuge	SD220	Clover
Micropipettes		Gilson, Labnet,
Micropipettes		Labmate, Nichipet,
Micropipettes		SOCOREX
Microtome		Reichert
Microtome blade		Reichert
Oven	240 litre	Binder
Orbital shaker	SH 30	FINEPCR
Orbital shaker	MS-OR	Major Science
Orbital shaking incubator	013422	Paton scientific
PCR	Master cycle	Eppendorf
PCR	PTC-200	MJ RESERCH

Instrument	Model	Company
pH meter	713	Metrohm
Power supply	ELITE 300 plus	Wealtec
Power supply	PAC 3000	Bio-Rad
Power supply	MP-300N	Major science
Surgical blade		Parabola
Slab gel electrophoresis	MINI PROTEIN II	BIO-RAD
Slab gel electrophoresis	MINI PROTEIN III	BIO-RAD
Slab gel electrophoresis	AE-6450	ATTO corporation
Spectrophotometer	8453	Hewlett-Packard
Spectrophotometer	Thermo spectronic	GENESYS 20
Spectrophotometer	UV-160A	Shimadzu
Stirrer		Corning
Vortex mixer	VX100	Labnet
Water bath		GallenKamp
Water bath	EcoTempTW20	Julabo
Water bath	WB-710M	OPTIMA

1.2 Chemicals

1.2.1 Analytical grade

Chemical	Company
Absolute ethanol	BDH
Acetic acid	Lab Scan
Acrylamide	Fluka
Agar	Merck
Ammonium persulphate	
Bis-acrylamide	Fluka

Chemical	Company
Boric acid	Merck
Calcium chloride	Merck
D-glucose	Univar
Dimethylsulfoxide (DMSO)	AMRESCO
Dipotassium hydrogen phosphate	Univar
Dithiothreitol	Bio-Rad
Ethylene diamine tetraacetic acid (EDTA)	Carlo
Formaldehyde	Sigma
Formamide	Sigma, AMRESCO
Glycerol	BHD, Univar
Hydrochloric acid	Merck
Methanol	Lab Scan, Merck
Phenol:chloroform:isoamyl alcohol (25:24:1)	Sigma
Silver nitrate	Carlo, Merck
Sodium acetate	CARLO EBRA
Sodium carbonate	Ajex, Merck
Sodium chloride	Lab Scan
Sodium dodecyl sulfate (SDS)	Finechem
Sodium hydroxide	Lab Scan
Sucrose	USB
Tris (Hydoxymethyl)- methylamine	USB
Tryptone	Merck
Tween Twenty (Tween 20)	APS Finechem
Xylene	Lab Scan
Yeast extract	Merck

1.2.2 Molecular biology grade

Chemical	Company
Agarose	Gene Pure LE
Ampicilin	Calbiochem
BamH I	GIBCO
Deoxynucleotide triphosphate	Promega
10 bp DNA ladder	invitrogen
100 bp DNA ladder	NEB Biolabs, Promega
EcoR I	GIBCO, NEB Biolabs
Ethidium bromide	Promega
Hha I	NEB Biolabs
Hind III	NEB Biolabs
Lambda DNA	NEB Biolabs
pGEM-T Easy	Promega
Proteinase K	Invitrogen
Sal I	NEB Biolabs
T4 DNA ligase	NEB Biolabs, Promega
T4 DNA kinase	Promega
Taq DNA polymeras	Invitrogen
X-gal	Promega

1.2.3 Reagent kits

Reagent	Company
Qiagen®PCR cloning kits	QIAGEN
QIA kit for plasmid purification	QIAGEN
QIA kit for PCR purification	QIAGEN
QIA kit for gel extraction	QIAGEN

1.3 Samples

1.3.1 Human Genomic DNA

Human genomic DNAs (250 samples) were provided by Rajanukul Institute, Bangkok, Thailand, under collaboration with DR. Verayuth Praphanphoj.

1.3.2 Embedded paraffin postmortem brain tissue

Human embedded parafilm postmortem brain tissues (20 samples) were provided by Chulalongkorn Hospital, Bangkok, Thailand.

1.3.3 Bacterial cells

E. coli DH5α is a gift from Professor Schreiber, Australia.

2. Methods

2.1 DNA preparation from paraffin-embedded brain tissue

DNA was extracted and purified from the paraffin-embedded brain tissues with the method described by Bonin *et al.* (2003). Ten of 10 μm-sections were cut with a microtomes (Reichert Microtome) from a paraffin wax block. To prevent cross contamination between samples, the microtome blade was cleaned after each block. The paraffin-embedded brain tissue (10 sections) was deparaffinized in a 1.5 ml-tube with 1 ml xylene at 55°C for 15 min. Removing of the paraffin was performed twice. The tissues was then washed for 15 min at room temperature with 100% ethanol and subsequently once with 70% ethanol. After air drying, the tissue pellets were digested with proteinase K (0.2 mg/ml) in 50 mM Tris/HCl, 1mM EDTA, and 0.5%Tween 20, overnight at 55°C. Then, DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and the clear aqueous phase was transferred to a new tube. To precipitate DNA, 0.1 volume of 3 M sodium acetate, pH 5.2, 2 volumes of ice-cold ethanol and 5% acrylamide solution (was used as a DNA carrier) was added and the solution was incubated overnight at -80°C. The DNA pellet was collected by centrifugation at 14,000 rpm for 20 min, and it was washed once with 70% ethanol, dried up and dissolved in 20 μl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The DNA were analyzed and determined for quality on 0.8% agarose gel stained with ethidium bromide prior to being stored at 4°C until used.

2.2 Amplification of ApoE and TTR gene fragments by polymerase chain reaction (PCR)

Specific oligonucleotide primer sets were used in amplifying DNA fragments of ApoE exon4, TTR exon1, 2 and 4 genes. The nucleotide sequences of the primers, as previous reported (Gioia *et al.*, 1998; Bonin *et al.*, 2003; Nichols and Benson, 1990), are shown in Table 2.1.

The PCR was performed using the purified human genomic DNAs as a template. The DNA (300 ng) was amplified in 100 µl of the reaction mixture containing buffer (20 mM Tris-HCl, pH 8.4 and 50 mM KCl), 250 mM $MgCl_2$, 25 mM dNTPs, 2.5 units of Taq DNA polymerase, and 25 pmol of each specific forward and reverse primers. For amplification of ApoE gene, 10% DMSO was additional added. PCR for the ApoE was started with an initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30s, annealing at annealing temperature (Ta) of the primers used (Table 2.1) for 30s and extension at 72°C for 30s. The final extension was carried out at 72°C for 5 min. To amplify the TTR gene fragments, PCR was carried out as following: denaturation at 94°C for 4 min, then 50 cycles of denaturation at 94°C for 1 min, annealing at annealing temperature (Ta) of the primers used (Table 2.1) for 1 min and extension at 72°C for 1 min. The final extension was carried out at 72°C for 10 min. The PCR products were analyzed by electrophoresis on 1% agarose and the DNA bands were visualized by staining with the solution of 0.4µg/ml ethidium bromide.

Table 2.1 Oligonuclectide primers used for amplification of TTR exon1, 2, 4, and ApoE exon4

PCR fragment	Fragment size (bp)	Type	Primer sequence (5' → 3')	Ta (°C)
TTR Exon1	291	forward	CAGCAGGTTTGCAGTCAGAT	55
		reverse	GGTACCCTTGCCCTAGTAAT	
TTR Exon2	311	forward	TCTTGTTTCGCTCCAGATTTC	58
		reverse	CAGATGATGTGAGCCTCTCTC	
TTR Exon4	258	forward	TAGGTGGTATTCACAGCC	60
		reverse	GTGCCTTTCACAGGAATG	
ApoE Exon4	300	forward	GGCACGGCTGTCCAAGGAGC	63
		reverse	ACGCGGCCCTGTTCCACCAG	

2.3 Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP)

Polymorphism of ApoE gene was determined as described by Hixson and Vernier (1990) and Wenham *et al.* (1991). The PCR products (400 ng) of ApoE exon4 gene were digested with 7.5 units of restriction enzyme *Hha*I (New England Biolabs) at 37°C for 6 h. The digested DNAs were separated in 20% polyacrylamide gel (prepared from a stock acrylamide solution containing ratio of acrylamide and bis-acrylamide at 38: 2) containing 10% glycerol, using the Mini PROTEIN II or III (Bio-Rad, Hercured, CA) and TBE buffer pH 8.3, at 4°C with a constant voltage (150 volts) for 6 h. The temperature of the system was kept cool throughout the separation. Thereafter, the DNA bands were visualized by staining the gel with silver nitrate.

2.4 Single-stranded conformation polymorphism (SSCP)

SSCP was performed as described by Connor *et al.*, 2004. In brief, genomic DNA was amplified for TTR exon 1, 2 and 4 by PCR using the specific nucleotide primer pairs (Table 2.1). Then, the PCR product (100 ng) was incubated with 2 volumes of the buffer containing 98% formamide, 10 mM NaOH, 10 mM EDTA, 0.05% xylene cyanol and 0.05% bromphenol blue, at 95°C for 10 min, before

the mixture was chilled on ice and analyzed on 20% polyacrylamide gel (prepared from a stock acrylamide solution containing ratio of acrylamide: bis-acrylamide at 29.2: 0.8) containing 10% glycerol. The electrophoresis separation was carried out in Mini PROTEIN II or III (Bio-Rad, Hercured CA) using TBE buffer pH 8.3 at 4°C with a constant voltage (150 volts) for 3 h. Temperature was kept cool through the run of gel. Thereafter, the gel was stained with silver nitrate.

2.5 Cloning of PCR product

Purified PCR product such as TTR gene fragment was ligated to the *E. coli* cloning vector, pGEM-T Easy (Promega) according to the method described by the company (Figure 2.1). In brief, the ligation reaction mixture (10μl in total volume), comprising of 20 ng of the purified DNA and 25 ng of the cloning vector, was incubated at 37°C for 2 min prior to chilling on ice and 2 units of T4 DNA ligase was added, and the incubation was continued at room temperature for 2 h. The reaction mixture was immediately transformed into a bacterial cell as described in section 2.7 or kept at 4°C until used.
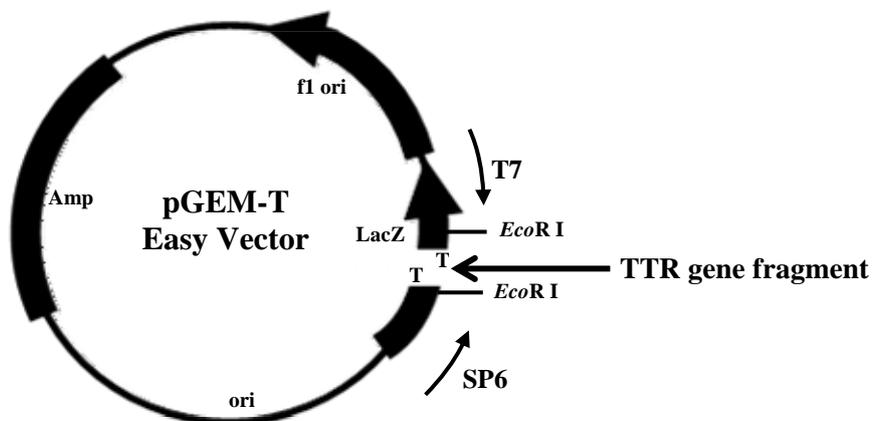
Figure 2.1   Cloning of TTR gene fragments for nucleotide sequencing

pGEM-T Easy and pDrive vector were selected as vehicles to bring TTR exon gene into and being amplified in the *E. coli* cell. The vectors contain origin of replication of the filamentous phage f1, and T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the α-peptide coding region of β-galactosidase. The DNA fragment was inserted into the vector between the *EcoR* I sites. Priming between the DNA of interest with the pGEM-T Easy and the pDrive vector is ensured by a single 3′-T and 3′-U overhangs at the insert site, respectively. f1 origin represences phage f1 origin; Amp is ampicillin resistance gene; kanamycin is kanamycin resistance gene; lacZ is lacz α-peptide coding region used in selection of the blue/white colony screening.

2.6 DNA sequencing

Nucleotide sequence of the DNA inserted plasmid or PCR product was determined by the Scientific Equipment Center, Prince of Songkla University (PSU) with the modified chain termination sequencing method of Sanger *et al*. (1977), using a Dye Terminator Cycle Sequencing Kit (Applied Biosystems). DNA was separated according to size in polyacrylamide gel under denaturation and the data processing was performed using the ABI Prism 377 Automated DNA sequencer (Applied Biosystems). The alignment program e.g. BLAST was used to check and compare the obtained nucleotide sequences.

2.7 Preparation of *E. coli* competent cells and transformation of DNA into cell

Competent cells were prepared from *E. coli* strain DH5α by using calcium chloride as described by Cohen *et al*. (1972). A single colony of the freshly overnight grown bacteria was picked up from the Luria-Bertani (LB) agar plate and inoculated in 5 ml of LB broth in a 50-ml tube at 37°C. Subculturing was performed with 50 µl of the overnight culture, and cells were grown until OD_{600} of the culture reached 0.4 to 0.5 (2 to 3 h). Then, cells were transferred to a polypropylene tube, cooled down on to 0°C and collected by centrifugation at 2,500 rpm for 10 min, at 4°C. All supernatant was removed and tube was inverted for 1 to 2 min to drain off all trace of the medium. The cell pellet was then suspended in ice-cold 0.1 M calcium chloride (10 ml of the solution per 50 ml of the original culture) and cooled down to 0°C. After removed out the salt solution by centrifugation, cells were resuspended in ice-cold 0.1 M calcium chloride (2 ml of the solution per 50 ml of the original culture). The competent cells were aliquoted and glycerol was added to the final concentration of 15.5% (v/v). Cells were quickly frozen in liquid nitrogen and immediately kept at -80°C as the glycerol stock.

To transform a foreign DNA into the competent cell, 50 µl of the competent cell suspension was mixed and incubated on ice for 10 min with the DNA (approximately 0.5 µg) or an aliquot (10 µl) of the ligation mixture in a polypropylene tube. Then the cell mixture was subjected to heat-shock at 42°C for exactly 90s without shaking prior to cooling down on ice for 1 to 2 min. Thereafter, SOC medium

(100 µl) was added and the mixture was incubated at 37°C for 1 h prior being plated onto a LB agar supplemented with X-gal (150 µg/ml agar) and ampicillin (100 µg/ml agar). The cells were grown overnight at 37°C. Five to ten single white colonies, which were expected to contain the DNA inserted plasmid, were selected for the plasmid purification.

2.8 Purification of PCR product

The DNA fragment amplified by PCR was purified by using the QIAquick PCR Purification Kit (Qiagen) and following the protocol described by the company. In brief, 5 volumes of the binding buffer containing guanidine hydrochloride were added to the reaction mixture, and the entire mixture was applied onto a spin column. The DNA bound to the column while other impurities were washed out with the buffer containing ethanol. In the final step, the plasmid was eluted out with 30 to 50 µl of 10 mM Tris-HCl, pH 8.5 and stored at -20°C.

2.9 Purification of DNA plasmid

2.9.1 By using the QIAprep Miniprep kit

Plasmid was isolated from bacterial cell by an alkaline lysis, using a QIAprep Spin Miniprep Kit (Qiagen) with a procedure recommended by the company (Birnboim and Doly, 1979). In brief, bacterial cells from 2 to 5 ml of an overnight culture with antibiotic were collected by centrifugation and resuspended in a buffer containing RNase A. Then, membrane of the cells was disrupted using an alkaline buffer for 5 min at room temperature. Thereafter, the cell lysate was neutralized and adjusted to be in a chaotropic salt (1.6 M guanidine hydrochloride) for binding to the silica material with a neutralizing buffer (4 M guanidine hydrochloride, 0.5 M potassium acetate, pH 4.2). The precipitates of chromosomal DNA and cell debris that occurred were removed by centrifugation then the supernatant was loaded onto a silica based membrane column. Plasmid was purified depending on its absorption to the surface of the silica filter and then was separated out from the unbound impurities in the washing step with a solution of 20 mM NaCl and 2 mM Tris-HCl, pH 7.5 in 80% methanol. Finally, the plasmid was eluted out from the column with 10 mM Tris-HCl, pH 8.5, and stored at -20°C until used.

2.9.2 By using a QIAquick PCR purification Kit

Plasmid in an enzymatic reaction was purified with the same method that was described for purification of the PCR product (section 2.8), using a QIAquick PCR Purification Kit (Qiagen).

2.10 Isolation and purification of DNA from agarose

DNA plasmid or PCR product that were separated on an agarose gel can be isolated and purified using a QIAquick Gel Extraction Kit (Qiagen), following the protocol of the company. After electrophoresis, the gel containing band of the DNA of interest was excised with a sterile razor blade. The gel slice was, then, dissolved in the solution containing guanidine thiocyanate with a ratio of gel : solution at 1 : 3 (v/v). Thereafter, 1 gel volume of isopropanol was added and the entire mixture was applied onto a QIA spin column. The DNA was adsorbed onto a membrane of the column while the impurities were washed out with a buffer containing ethanol. Finally, the DNA was eluted with an appropriate volume of 10 mM Tris-HCl, pH 8.5 and stored at -20°C until used.

2.11 Restriction analysis of DNA inserted plasmid

To determine size of the inserted DNA, the recombinant plasmid was digested with an appropriated restriction endonuclease. In general, the enzymatic reaction mixture (20 μl) was comprised of 200 to 5,000 ng of plasmid and 10 units of each appropriate enzyme. The reaction was then carried out at 37°C for 1.5 to 2 h prior analysis by agarose gel electrophoresis was performed and DNA bands were visualized by staining with ethidium bromide.

2.12 Determination of DNA concentration

Concentrations of the DNA samples and oligonucleotide primers were determined by spectrophotometric absorption at wavelength 260 nm. One OD_{260} was assumed corresponding to 50 μg/ml of double stranded DNA or 33 μg/ml of oligonucleotide. Purity of the DNAs was determined from the ratio of OD_{260}/OD_{280}

and the ratio in range of 1.8 to 1.9 indicating good purity of the DNAs (Sambrook *et al.*, 1989).

2.13 Non-denaturing PAGE

The analysis of DNA fragments under non-denaturing condition was performed using vertical polyacrylamide slab gel (20% resolving gel and 4% stacking gel) and TBE buffer. For a particular work, glycerol was added to a final concentration of 10% to both resolving and stacking gels. After the separation, DNA bands were detected by silver staining.

2.14 Silver staining

Polyacylamide gel staining with silver nitrate was performed using the method of Morrissey, 1981. After electrophoresis, gel was placed in a solution containing 9.2% acetic acid and 45.4% methanol for 30 min and transferred to a solution containing 7.5% acetic acid and 5% methanol for 30 min. Then, it was rinsed with distilled water for 3 to 4 changes prior to being soaked in the solution of DTT (500 µg/ml) for 30 min. Thereafter, the solution was discarded and gel was soaked in 0.1% silver nitrate for 20 min. To develop the gel, the solution containing 3% (v/v) sodium carbonate and 0.02% (v/v) formaldehyde was added, and gel was soaked until bands appeared. To stop the reaction, 50% acetic acid was added until air bubbles occurred. Gel was washed several times with distilled water prior being stored at 4°C in a plastic bag. The gel was photographed by gel document (Labworks 4.0, Bio-Rad).

2.15 Lambda DNA/Hind III marker preparation

The Lambda/Hind III marker was prepared by digestion of 10 µg Lambda DNA with 10 units of Hind III (NEB Biolabs). The DNA bands were determined for quality by separation on 0.8% agarose gel staining with ethidium bromide.

CHAPTER 3

RESULTS AND DISCUSSION

1. DNA preparation from paraffin-embedded brain tissue

Paraffin wax embedded tissues are an extraordinary source for DNA molecular studies because of the availability of large pathology archives of tissues related to clinical cases in almost all hospital pathology departments. In addition to biopsy and surgical paraffin wax embedded tissues, postmortem tissues are an important resource, especially for rare diseases, for neuropathology studies or molecular epidemiology studies. However, the major difficulty in using the tissues is the degradation of nucleic acids, which is more extensive than in paraffin wax embedded tissue of biopsies or surgical specimens.

In this thesis, genomic DNAs were succseefully prepared from the paraffin-embedded brain tissue of 20 Thais people whom have been diagnosed for the Alzheimer disease. Analysis on agarose gel of the DNA isolated from the individual sample showed size of the DNAs less than 23 kb, comparing to the lamda DNA marker (Figure 3.1). From the Human Genome Project that shotgun sequencing was used to generate and the whole consensus sequence of the euchromatic portion of human genome was determined, it revealed that size of the human genome is more than 3.2 billion base pairs (Venter *et al*., 2001). Therefore, size of the genomic DNA purified from the paraffin-embedded brain tissue obtained in this thesis indicating the degradation of the DNA during the tissue processing, which is a source of great variability occurred from the routine clinical laboratories without completely being standardized. These variability include type of the fixative solution, storage conditions (especially when formaldehyde solution is used), and suppliers. Fixation and storage tissues in the formaldehyde solution, which is routine used, for more than one week has been reported for nucleic acids damages as the chemical induces extensive cross-linking of the tissue proteins leading to nucleic acid fragmentation (Lehmann *et al*., 2001). Tissues are suggested to be fixed in a buffered formaldehyde solution at pH in a physiological range in dark for 24 h before embedding in the paraffin wax. More extensive degradation of DNA is usually found in the postmortem tissues because

these tissues are usually fixed for a long period and in the non-buffered formaldehyde solution, which always used in the past. In the non-buffered solution, oxidation of formaldehyde to formic acid occurs creating an acidic environment, which is the main cause of the DNA degradation. Although DNA is relatively stable in mildly acidic solutions, protonation of the purine bases (N7 of guanine and N3 of adenine) occurs in the acidic environment in particular at around pH 4, leading to hydrolysis of the β-glycosidic bond of the purine bases and easily cleavage of the purines. Once this occur, the depurinated sugar can isomerize into the open chain form thus increase susceptibility to be cleavage by hydroxyl ions of the depurinated or apurinic DNA (Voet *et al.*, 1999). In average, size of the DNA isolated from the biopsy tissues is 300-400 bp, but much shorter can obtain from the postmortem paraffin wax embedded tissues (Lehmann *et al.*, 2001; Ninet *et al.*, 1999).

In this thesis, the spectrophotometric measurement of the absorbance at wavelengths of 260 nm and 280 nm was used to estimate the purity of the genomic DNA purified from the embedded tissues, which might have some contaminants such as proteins and phenol those also have the absorbance at wavelength 280 nm. A260 and A280 are frequent used to determine the concentrations of nucleic acid and protein, respectively. The ratio of A260/A280 less than 1.5 suggests protein contamination in a nucleic acid sample (Sambrook *et al.*, 1989). The ratio of A_{260}/A_{280} of the DNA samples obtained in this thesis were ~1.1 to 2.2 (data not shown). These might indicate contamination of some proteins as well as phenol in the DNA samples. However, as physicochemical alterations produced by pH and temperature, and chemical modification can also increase the absorbance ratio of 260/280 nm to more than the normal range (1.8-1.9) (Wilfinger *et al.*, 1997; Okamoto and Okabe, 2000; Kim *et al.*, 2005), the quality of DNA was also assessed by agarose gel electrophoresis and its performance in PCR was evaluated. The analysis on agarose gel electrophoresis can inform not only average size, but also degradation degree of the DNA (Figure 3.1).
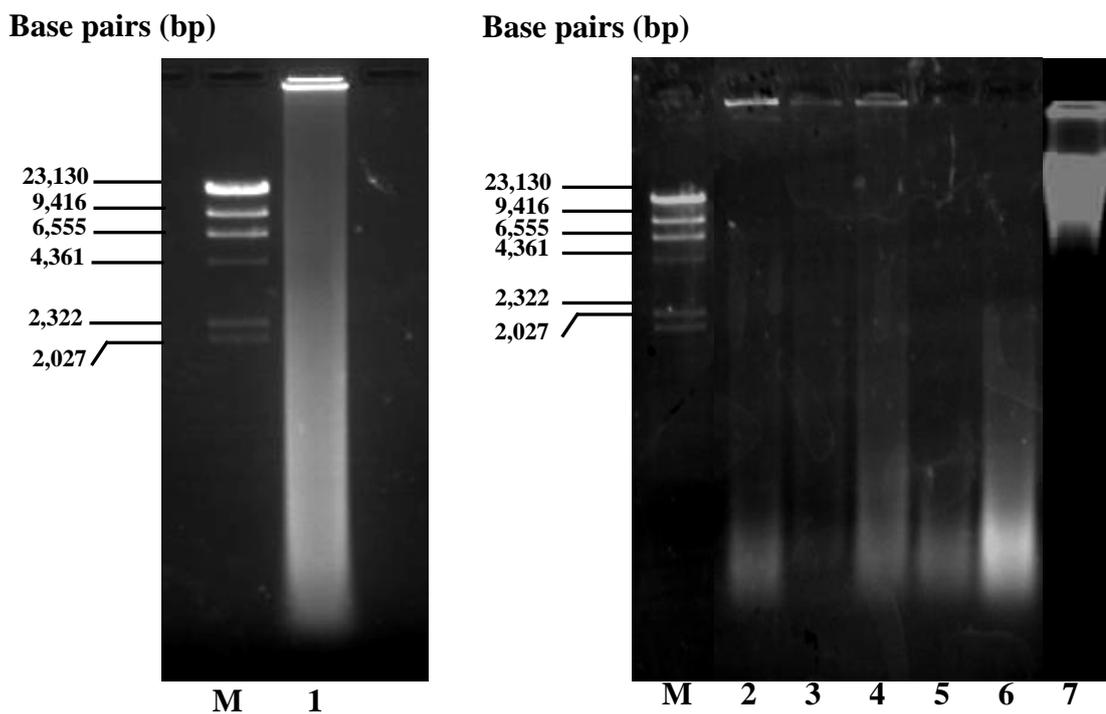
Figure 3.1   **Mobility of human total genomic DNA on agarose gel**

Total genomic DNA (~1μg) was separated on 0.8% agarose. M, the DNA marker (23 kb Lambda DNA/Hind III); 1 to 6, the DNA purified from the individual paraffin-embedded brain tissue; 7, genomic DNA purified from blood. Degradation of the DNA to a smear zone was observed in all samples obtained from the embedded tissues but not from the fresh blood.

2. Apolipoprotein E genotyping by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP)

The gene of ApoE is located on chromosome 19 containing four exons and spanning to 3.7 kb (Olaisen *et al.*, 1982). Three common isoforms of ApoE, E2, E3 and E4, are encoded by allele ε2, ε3 and ε4 (Zannis and Breslow, 1981). These isoforms differ from each other at codons 112 and 158, which are encoded by exon 4 of the ApoE gene. E2 has cysteine at position 112 and 158, while E3 has cysteine at position 112 and arginine at position 158, and E4 has arginine at both positions. The ApoE-ε3 is the most common allele in population with the frequency of about 78% in European and American Caucasian whereas ApoE-ε4 and ε2 represent approximately 15% and 7% of all alleles, respectively. These 3 alleles are expressed co-dominantly to generate six different genotypes. While it has been reported that the ε4 allele of ApoE is associated with earlier age at the onset and increased risk of Alzheimer's disease (AD), the ApoE-ε2 allele may reduce the risk of dementia in heterozygous carriers.

In this thesis, the ApoE exon 4 was amplified from the genomic DNA purified from the peripheral blood of 237 individual Thai people by PCR under conditions as described in section 2.2. The specific oligoprimers containing nucleotide sequences as shown in Table 2.1 were used to amplify fragments of the gene. An example of the analysis on 1% agarose gel of the PCR products was shown in Figure 3.2. Only a single band of the DNA fragment was detected from each reaction. The fragment sizes of the DNA were similar to that previously reported (Lynda *et al.*, 1998), i.e. 300 bp. Specificity of the amplification reactions was very high, evidently from high intensity of the amplified DNA bands obtained. These confirmed good quality of the purified DNA templates.

Nowadays, genotyping of the apolipoprotein E by PCR-RFLP has been widely replaced the isotyping by gel electrophoresis. The molecular analysis is performed using a pair of specific primers to amplify region in the ApoE exon 4 that encodes and generates polymorphisms characteristic of the three common ApoE alleles. Restriction endonuclease *HhaI* is used to generate the DNA fragments of typical lengths for the ε2, ε3 and ε4 alleles. Because arginine (CGC) substitution by

cysteine (TGC) at codons 112 and 158 alters the *Hha*I (GCGC) cleavage sites, all six common genotypes can be distinguished by the unique combinations of the different-sized restriction fragments.

In this thesis, the ApoE gene encompassing the nucleotide substitutions at residues 112 and 158 can be amplified by PCR with specific primers allowing each ApoE genotype to be identified as a specific combination of the DNA band patterns. The four *Hha*I digested fragments i.e. 91, 83, 72 and 48 bp, which are specifically used to differentiate six different ApoE genotypes, were clearly separated as distinct bands by 20% non-denaturant polyacrylamide gel electrophoresis as shown in Figure 3.3. Examples of the electrophoretic mobility patterns of the *Hha*I digested fragments of the ApoE exon 4 amplified from 48 individual normal people (average age is over 60 years; used as control), 33 patients with Down's syndrome (DS) (average age is 12.64 ± 5.57 years; 19 males and 14 females), 55 cases with other AD high risk (ADHR) (average age is 46.78 ± 11.45 years; 19 males and 36 females) and 101 individuals of ADHR control (used as the control for the ADHR) (average age is 45.31 ± 10.81 years; 31 males and 70 females) were shown in Figure 3.4. All of these individual were diagnosed of diseases following standard clinical criteria. The distribution of ApoE allele and ApoE genotypes among the study groups is shown in Table3.1.

In the normal group (n=48), the ApoE genotyping showed that distribution of homozygous ε3/ε3, heterozygous ε3/ε4, heterozygous ε2/ε3, homozygous ε4/ε4 and heterozygous ε2/ε4 was 52.08%, 25%, 12.5%, 2.08% and 8.33%, respectively. However, there was no ε2/ε2 genotype found in this study (Table 3.1). The allele frequencies of ε2, ε3 and ε4 for this group were 0.10, 0.71 and 0.19 respectively (Table3.1).

The ApoE genotyping of the DS patients (n=33) showed 60.61% of ε3/ε3, 18.18% of ε3/ε4, 15.15% of ε2/ε3 and 6.06% of ε4/ε4, whereas, ε2/ε2 and ε2/ε4 genotype was not found (Table 3.1). The allele frequencies of ε2, ε3 and ε4 for this group were 0.08, 0.77 and 0.15, respectively, (Table3.1). In comparison, the distribution frequency of the ε4/ε4 in Thai DS patients was triple to that of the normal group and, interestingly, there was no ε2/ε4.

The distribution of ApoE genotypes in the Thai ADHR control (n= 101) was 75.25% for ε3/ε3, 4.95% for ε3/ε4, 16.83% for ε2/ε3, 0.99% for ε4/ε4 and 1.98% for ε2/ε4 (Table 3.1). In comparison, distribution of the heterozygous and homozygous ε4, i.e. ε2/ε4, ε3/ε4 and ε4/ε4 in the ADHR control were much less than that in the normal group. However, similar to the normal group, the ε2/ε2 genotype was not found in these ADHR control (Table 3.1), and the allele frequencies of ε2, ε3 and ε4 were 0.09, 0.86 and 0.05, respectively, (Table3.1), which were similar to the normal group except the frequency of ε4 was almost 4 times less.

Genotyping of the ApoE in the Thai ADHR patients (n=55) revealed that 67.27% was ε3/ε3, 12.70% was ε3/ε4, 14.55% was ε2/ε3, 3.64% was ε4/ε4, 1.82% was ε2/ε4, and ε2/ε2 genotype was not found in this group (Table 3.1), whereas allele frequencies of ε2, ε3 and ε4 in this group were 0.08, 0.81 and 0.11 respectively (Table3.1). In comparing to the ADHR control group, the ADHR has double in frequency of ε4 and triple in distribution frequency of ε4 associated genotype i.e. ε3/ε4 and ε4/ε4, but not ε2/ε4.

The frequency of the ApoE genotypes as shown in Table 3.1 indicated that the ε3/ε3 was the most common ApoE genotype found in all of four Thai groups studied in this thesis. In addition, the result showed that the most common allele of these Thai groups was ε3. The finding that ε3/ε3 was the most common genotype of the Thai groups is similar to that previous reported in both healthy and elderly DS of other ethnics (Cosgrave *et al*., 1996; Seet *et al*., 2004). The frequencies of the other three genotypes i.e. ε2/ε3, ε2/ε4 and ε3/ε4 also were not much different from previous observed in other ethnics (Lambert *et al*., 1996; Seet *et al*., 2004; Coppus *et al*., 2007). The absence of the homozygous ε2/ε2 in all Thai groups was unexpected particularly when this genotype has been confirmed associate with longevity (Royston *et al*., 1994). The ε2/ε2 genotype was expected to be presence at least in the normal or the ADHR control group. Although the data obtained here differs from that previously reported for other ethnics and Thai healthy (Chanprasertyothin *et al*., 2000), it is similar to that found in Thai elderly with and without dementia (Senanarong *et al*., 2001). This should be convincible that the ε2 homozygous may protect the

development of dementia only in DS and elderly. However, effect of the sample size is also possible.

The apolipoprotein E gene polymorphism has been well known as a risk factor for AD and associated with the early onset of the disease (Strittmatter and Roses, 1995; Slooter *et al*., 1998). In comparison to other ethnics, the Thai normal group examined in this research has similar frequency of ε4/ε4 (Table 3.1). However, the ε4 homozygous in the DS group was three times in frequency to that in the normal (Table 3.1). This was different from the findings in the DS from other ethnics to which showed similar or less ε4 homozygous frequency than the control (Avramopoulos *et al*., 1996; Cosgrave *et al*., 1996; Lambert *et al*., 1996). According to the association of ApoE with AD, it should convince that this Thai DS group was developed AD faster than DS from the other ethnics.

The ADHR control and ADHR showed similar frequencies of ε2/ε3, ε2/ε4, ε3/ε3, ε2 and ε3 to other ethnics and also to the normal and the DS groups studied here. However, the difference in frequency was observed in ε3/ε4, ε4/ε4 and the ε4. The ADHR showed three times higher in frequency of ε3/ε4 than the ADHR control, whereas the frequency of ε3/ε4 in DS was lower than the normal group. The incident of ε4/ε4 in the ADHR was three times higher than in the control, ADHR control, similarly to that found in the DS. In addition, the frequency of the ε4 allele was twice of the ADHR control. These may suggest to high risk in development of dementia and AD in the ADHR group. However, the medical follow up as well as obtaining data from larger sample size are important for the accuracy in the statistically interpretation. In comparison to the DS from many ethnics (Royston *et al*., 1994; van Gool *et al*., 1995; Wisniewski *et al*., 1995; Martin *et al*., 1995; Lambert *et al*., 1996; Tyrrell *et al*., 1998; Deb *et al*., 2000; Anello *et al*., 2001; Coppus *et al*., 2006), the Thai DS showed similar allele frequency and genotypes (Table 3.2).

Base pairs (bp)
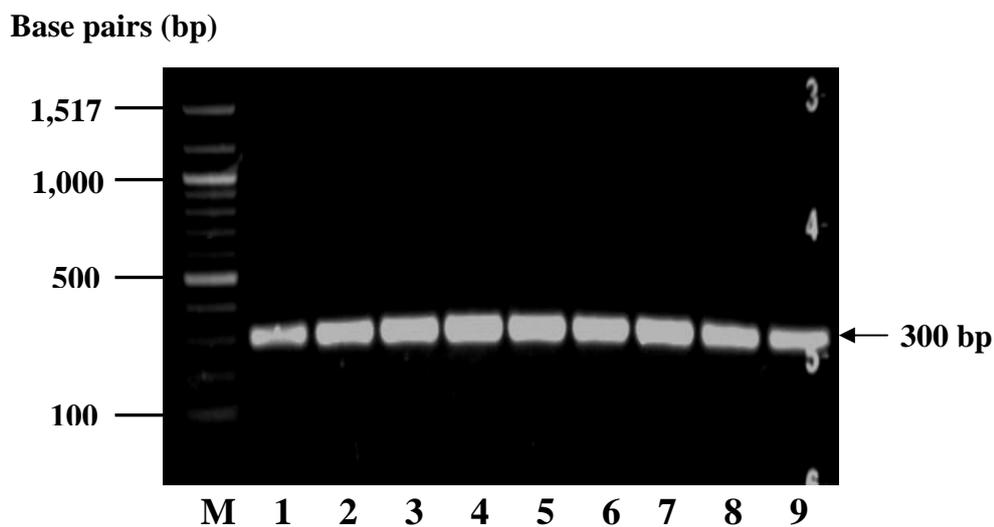
Figure 3.2 **Amplification of ApoE exon4 by PCR**

PCR was used to amplify ApoE exon4 from human genomic DNA as described in section 2.2. The PCR products were separated by electrophoresis on 1% agarose gel containing ethidium bromide, and the DNA fragment bands were visualized under UV. M is the DNA marker (100 bp ladder). 1 to 9 is the PCR product amplified from an individual human genomic DNA sample.

Base pairs (bp)
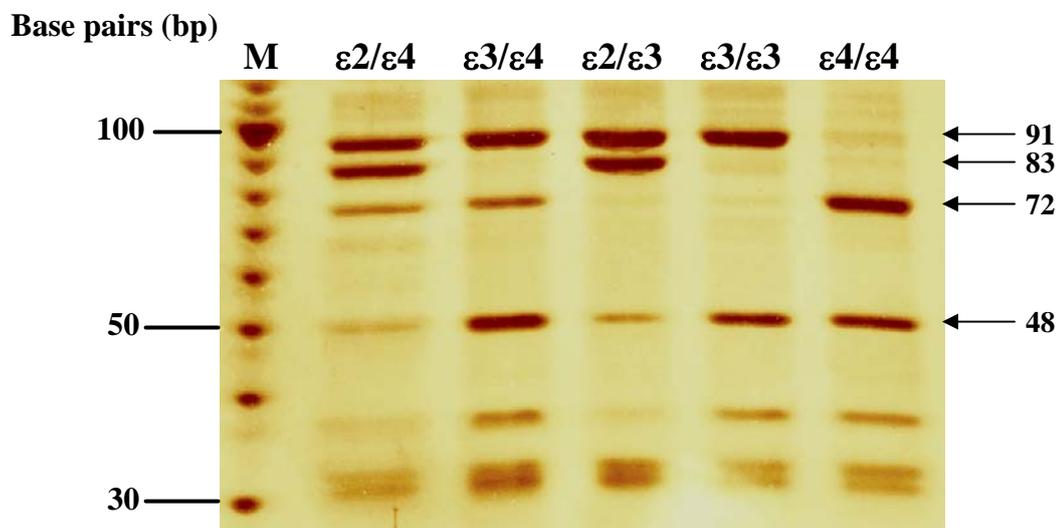
Figure 3.3   Polyacrylamide gel patterns of ApoE genotypes

The ApoE gene that encompasses the nucleotide substitutions at the amino acid residues 112 and 158 can be amplified by PCR using specific primers and after analysis on polyacrylamide gel, each ApoE genotype can be identified as a specific combination of the DNA band patterns. Five genotypes i.e. ε2/ε4, ε3/ε4, ε2/ε3, ε3/ε3 and ε4/ε4, but not ε2/ε2, can be detected in this research. After PCR, the PCR product of the ApoE gene (400ng) was digested with *HhaI* prior to analysis by native PAGE (20% resolving gel). The DNA band fragments were visualized by silver staining as described in sections 2.4 and 2.14. Arrows indicate positions of the DNA fragments (91, 83, 72 and 48 bp) generated from the enzymatic reaction and used to identify the allele. M is the DNA marker (10 bp ladder).

A

Base pairs (bp)

B

Base pairs (bp)

C
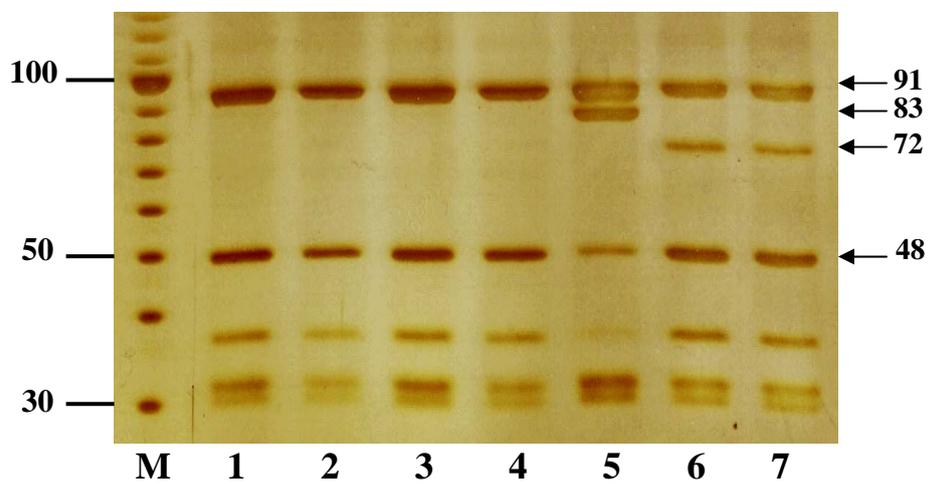
Base pairs (bp)
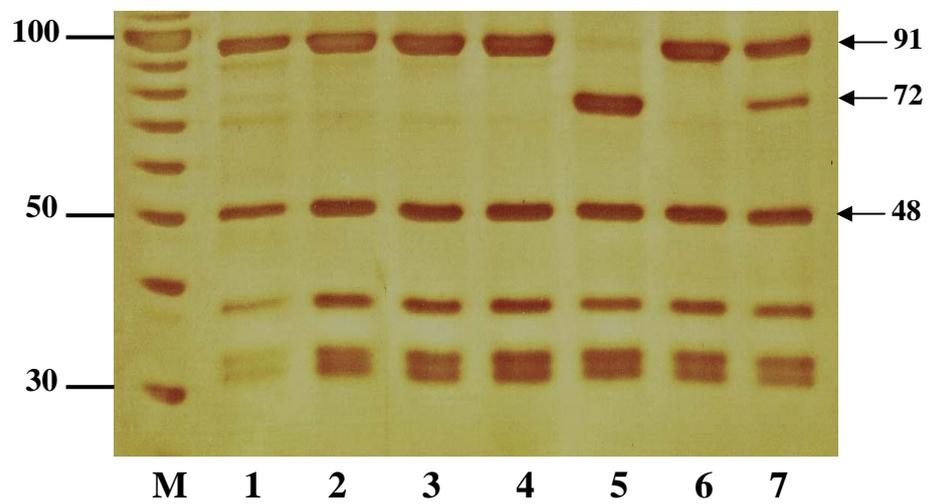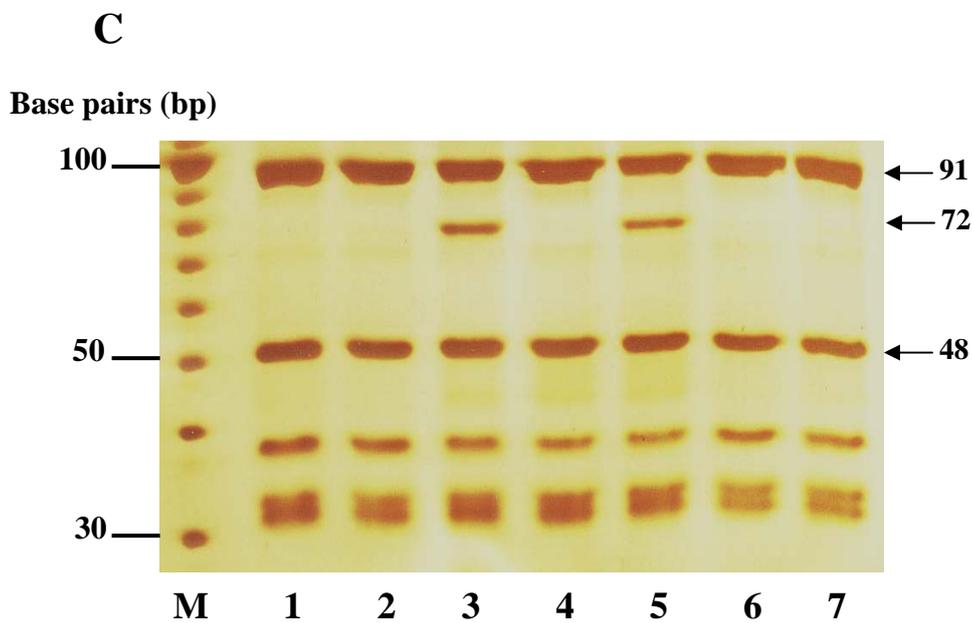
Figure 3.4   **ApoE genotyping of the Thai people groups**

PCR-RFLP analysis was performed to determine the genotype of ApoE. Only 5 ApoE genotype i.e. ε2/ε4, ε3/ε4, ε2/ε3, ε3/ε3 and ε4/ε4 were detected in groups of normal (A), DS (B), and ADHR control and ADHR (C), that were studied in this thesis. Positions of the DNA fragments (91, 83, 72 and 48 bp) used for the allele identification are indicated by arrows. 1 to 7 indicates individual samples. M is a DNA marker (10 bp DNA Ladder).

Table 3.1   Distribution of the ApoE alleles in the Thai groups

Group	Age (mean±SD)	Male/Female	Allele frenquency (%)			Genotype distribution (frequency in %)					
			ε2	ε3	ε4	ε2/ε2	ε2/ε3	ε2/ε4	ε3/ε3	ε3/ε4	ε4/ε4
DS (n=33)	12.64 ± 5.57	19/14	7.58	77.28	15.15	0	15.15	0	60.61	18.18	6.06
Normal (control) (n=48)	60±	22/26	10.42	70.83	18.75	0	12.50	8.33	52.08	25.00	2.08
ADHR (n=55)	46.78 ± 11.45	19/36	8.20	80.90	10.90	0	14.55	1.82	67.30	12.70	3.64
ADHR control (control for ADHR) (n=101)	45.31 ± 10.81	31/70	9.40	86.14	4.46	0	16.83	1.98	75.25	4.95	0.99

Table 3.2 **Comparison of the ApoE allele frequencies in DS**

Study		n^a	Age^b	Allele frequency			Genotype distribution					
				ε2	ε3	ε4	ε2/ε2	ε2/ε3	ε3/ε3	ε3/ε4	ε4/ε4	ε2/ε4
Royston *et al.* (1994)	DS	44	±62.4	0.27	0.62	0.12	1	4	9	8	-	-
Van Gool *et al.*(1995)	DS	104	51.9±6.2	0.09	0.80	0.12	1	6	33	11	-	1
Wisniewski *et al.* (1995)	DS	38	±49.3	0.11	0.89	-	-	3	16	-	-	-
Martin *et al.* (1995)	DS	46	±46	0.12	0.70	0.18	-	6	13	3	-	-
Lambert *et al.* (1996)	DS	82	45.2±7.7	0.11	0.74	0.15	-	7	23	8	1	2
Tyrrell *et al.* (1998)	DS	442	47.1±8.2	0.05	0.82	0.13	2	17	151	42	5	4
Deb *et al.* (2000)	DS	114	51±7.8	0.03	0.85	0.12	-	3	40	14	-	-
Anello *et al.* (2001)	DS	120	22.1±10.5	0.03	0.92	0.04	-	4	51	5	-	-
Coppus *et al.* (2007)	DS	850	51.9±6.2	0.11	0.73	0.16	7	59	243	88	10	-
This thesis	**DS**	**66**	**12.64±5.57**	**0.08**	**0.77**	**0.15**	**-**	**5**	**20**	**6**	**2**	**-**

[a]Number of alleles; [b]mean±S.D.; DS, Down's syndrome

3. SSCP of TTR gene

Screening for the point mutation of TTR gene was performed by SSCP technique, which is one of the effective methods for screening and identification of the sequence variations in a DNA single strand. The technique was first announced in 1989 as a tool for detecting DNA polymorphisms, or sequence variations. The analysis offers an inexpensive, convenient, and sensitive way for determining genetic variation. Similar to restriction fragment length polymorphisms (RFLPs), SSCPs are allelic variants of inherited, genetic traits that can be used as genetic markers. However, unlike RFLP, the SSCP analysis can detect DNA polymorphisms and mutations at multiple places in DNA fragments (Orita *et al.*, 1989). For the medical diagnoses, SSCP analysis is often used as a mutation scanning method to detect for the polymorphisms at single loci (Sunnucks *et al.*, 2000). Within the SSCP technique, DNA is separated into single-strands and based on subtle differences in sequence, which is often a single base pair, will results in a different secondary structure and a measurable shift in mobility through a gel of the DNA single-strands. Migration of double-stranded DNA in gel electrophoresis is depended on size and length of the DNA strand and relatively independent on particular nucleotide sequence. On the other hand, very small changes in sequence, even one changed nucleotide out of several hundreds, can affect the mobility in gel of the DNA single strands. A single point mutation of TTR gene can be identified by this technique because of the relatively unstable nature of the single-stranded DNA. In the absence of a complementary strand, the single strand may undergo intra-strand base paring, resulting in loops and folds that give the single strand a unique three dimension structure, regardless of its length. A single nucleotide change could dramatically affect the strand's mobility through a gel by altering the intra-strand base pairing and its three dimension conformation (Melcher, 2000). SSCP analysis takes advantage of this quality of single-stranded DNA.

In this thesis, the TTR exon1, 2 and 4 were amplified by PCR using the purified genomic DNA extracted from 173 Thai participants as templates with conditions as described in section 2.2. By the standard phenol/chloroform extraction, the genomic DNA obtained from whole blood and lymphoblast showed much higher in quality (OD_{260}/OD_{280} ~1.3-2.0) than that extracted from the embedded paraffin

brain tissue (OD_{260}/OD_{280} ~1.1-2.2). However, all these DNAs could be used as templates for the PCR with the specific oligonucleotide primers containing sequences as shown in Table 2.1. The TTR exon1 was amplified from 47 Thai normal and 35 DS patients, while TTR exon2 and exon4 were amplified from ADHR control and ADHR (TTR exon2 from 59 ADHR control and 29 ADHR; TTR exon4 from 20 ADHR control and 13 ADHR). In addition, TTR exon1 and exon4 were attempted to being amplified from the DNAs extracted from the paraffin-embedded brain tissues.

By PCR, a single band of the DNA fragment from each reaction was detected after analysis on 1% agarose gel (Figure 3.5 and 3.6). The sizes of the DNA fragments were 291bp, 311bp and 258bp for the TTR exon1, 2 and 4, respectively, which were well agree with that reported previously (Bonin *et al*., 2003; Nichols and Benson, 1990). By SSCP analysis, the mobilities of most TTR exon fragments were similar among the normal, the DS, the ADHR control and the ADHR groups (Figure 3.7 A-D). However, changes in mobility of TTR exon4 were detected in one ADHR control and one ADHR (Figure 3.7 B, C). In addition, a change in mobility of the TTR exon1 was observed in one DS and two normal (Figure 3.7 D). Double rather a single band was detected. All of these abnormal double DNA bands showed different mobilities i.e. one moved with the normal rate while the other moved slower or faster. The abnormality was confirmed to be resulted from a single mutation of the TTR gene by nucleotide sequencing.

In this thesis, the electrophoretic mobility of the TTR exon1 and exon4 amplified from the DNA extracted from the paraffin-embedded brain tissue was compared to those amplified from whole blood or lymphoblast cell lines (Figure 3.7 E). However, no difference in mobility of the TTR exons could be observed.

A

Base pairs (bp)

B

Base pairs (bp)

C
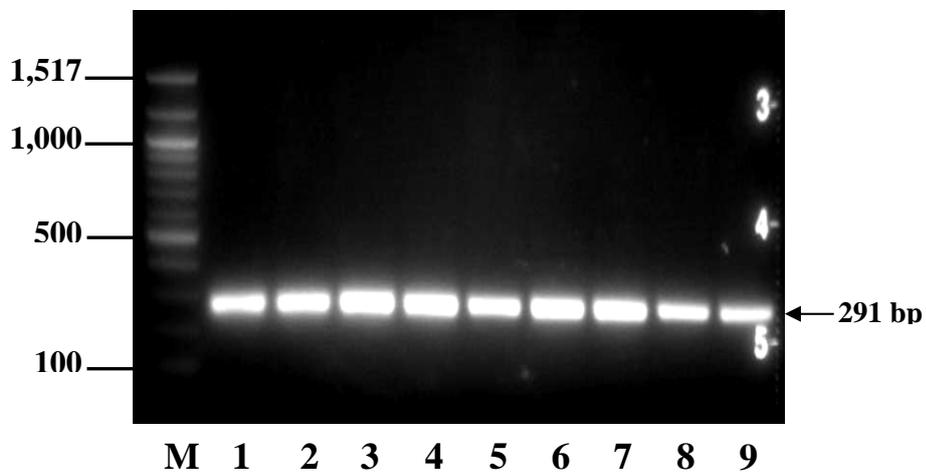
Base pairs (bp)
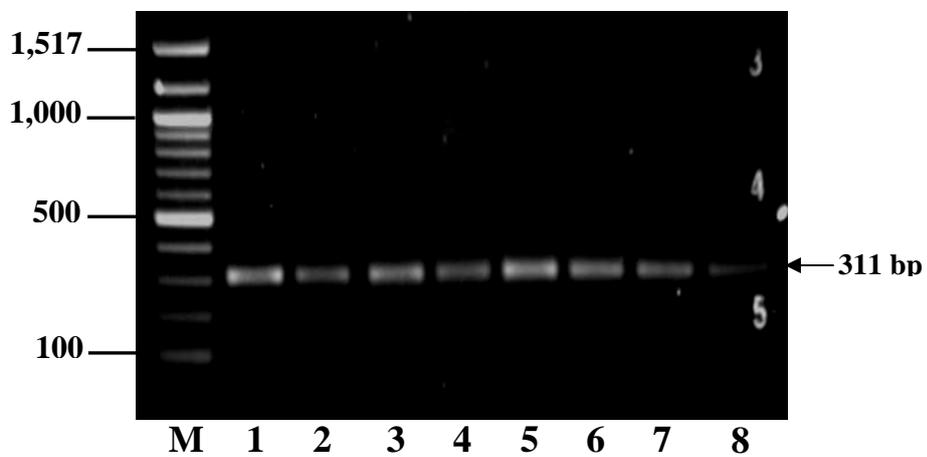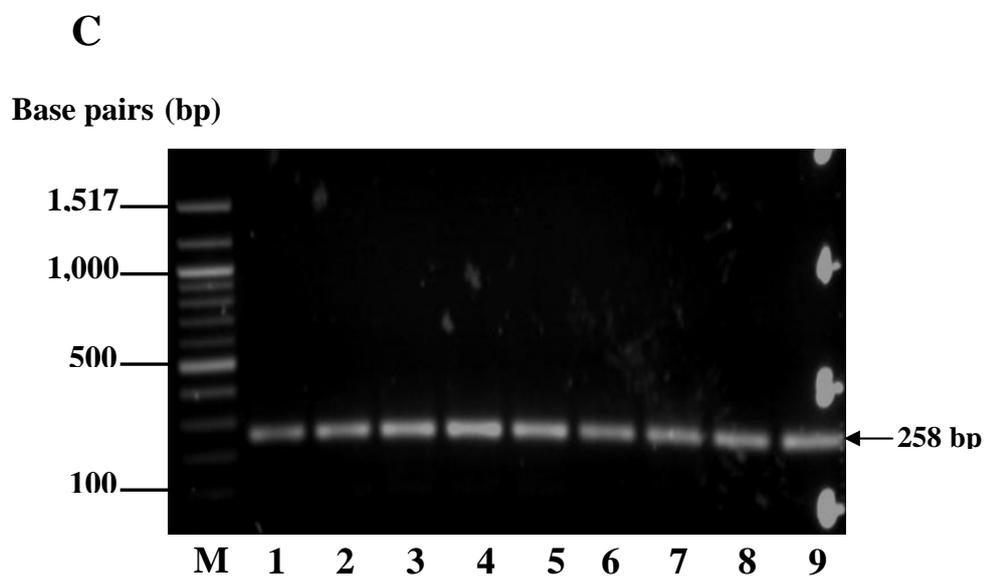
Figure 3.5 **Amplification of TTR gene fragments from the whole blood and lymphoblast DNAs**

PCR was used to amplify TTR exon1 (A), exon2 (B) and exon4 (C) from human genomic DNA that was extracted from whole blood or lymphoblast. The PCR products were then analyzed by electrophoresis on 1%agarose gel containing ethidium bromide, and the DNA bands were visualized under UV. M is a DNA marker (100 bp ladder). 1 to 8 or 1 to 9 is the PCR product amplified from an individual genomic DNA sample.

Base pairs (bp)
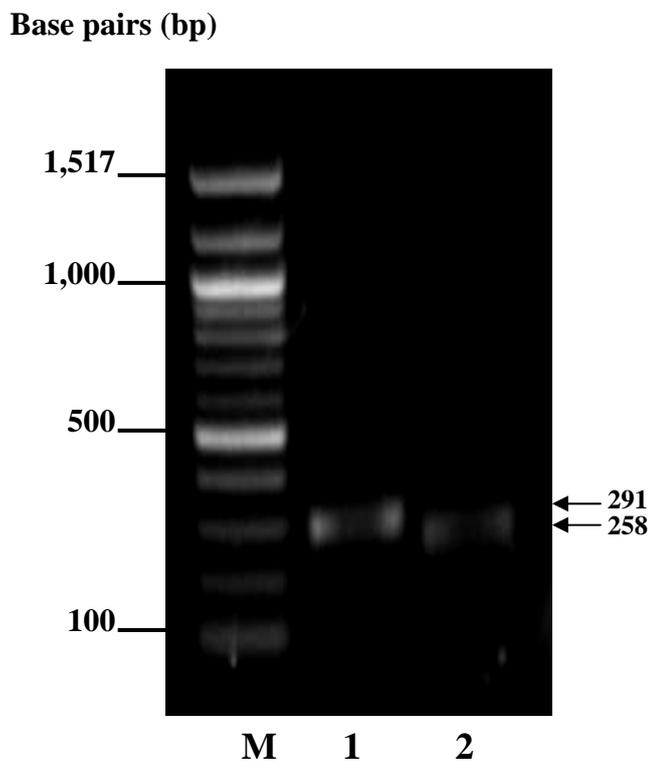
Figure 3.6   Amplification of TTR gene fragments from brain DNA

TTR exon1 and exon4 were amplified from the genomic DNA isolated from brain tissue by PCR. The PCR products were analyzed by electrophoresis on 1%agarose gel containing ethidium bromide, and the DNA bands were visualized under UV. M is a DNA marker (100 bp DNA ladder). 1 and 2 is the TTR exon1 and 4 that amplified from the same genomic DNA sample.

A

B

C

D

E

M   1   2   3   4
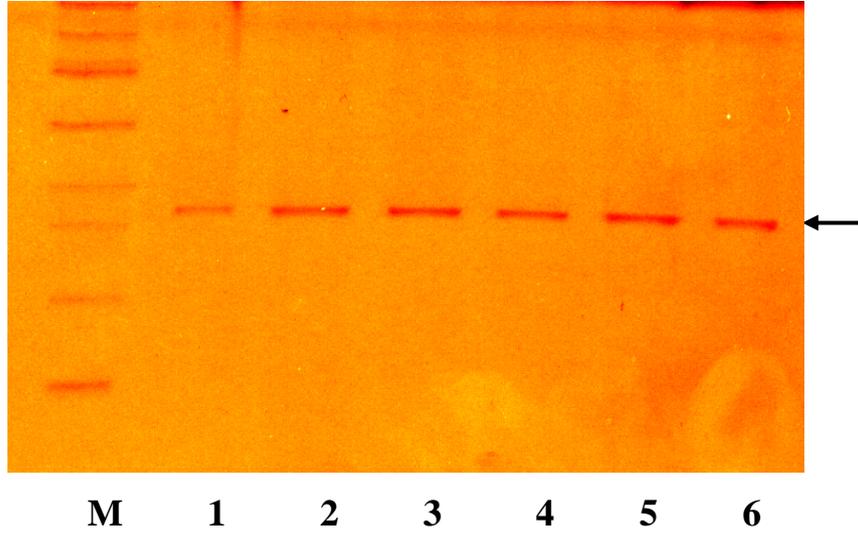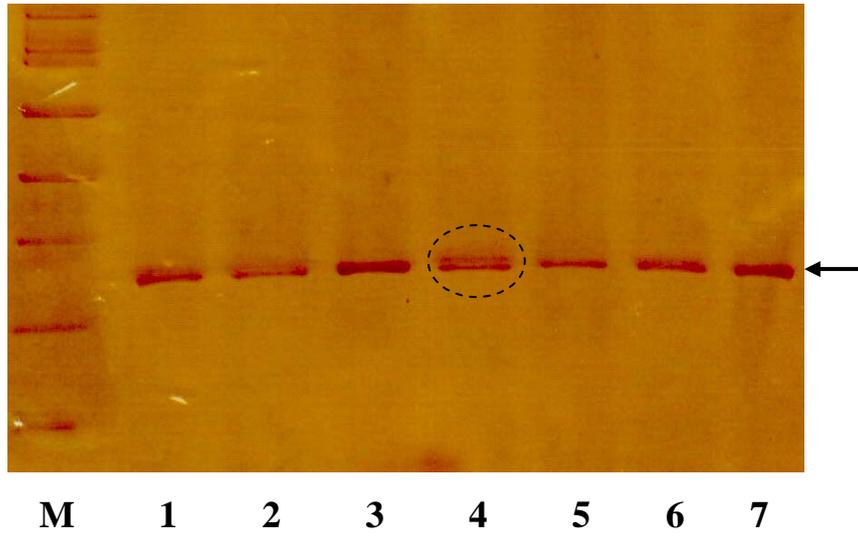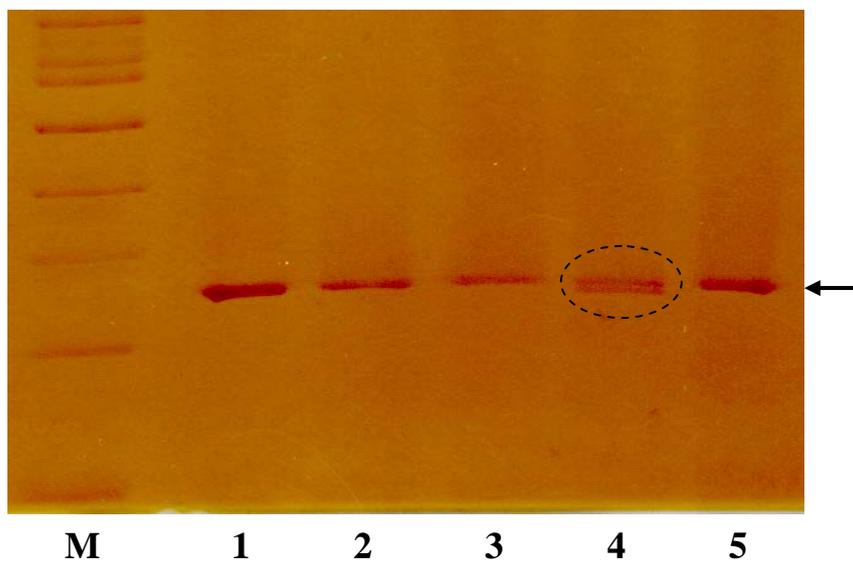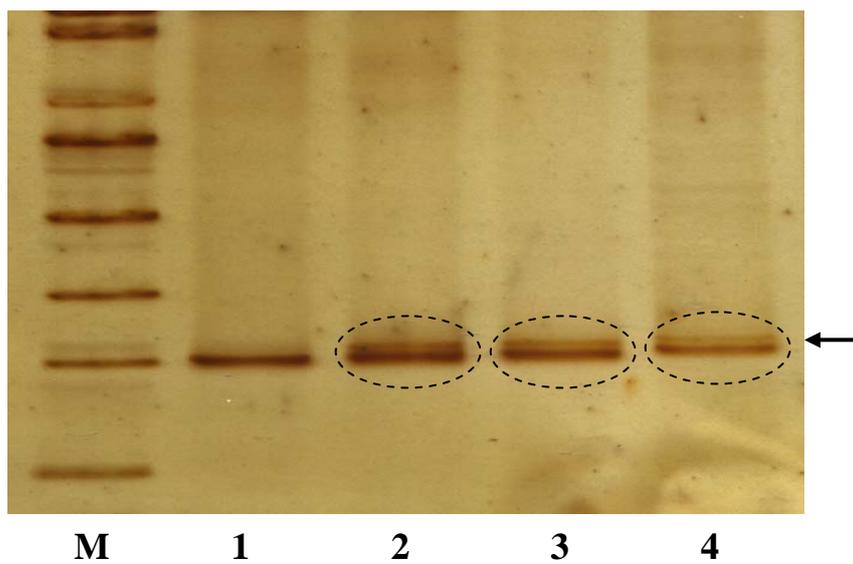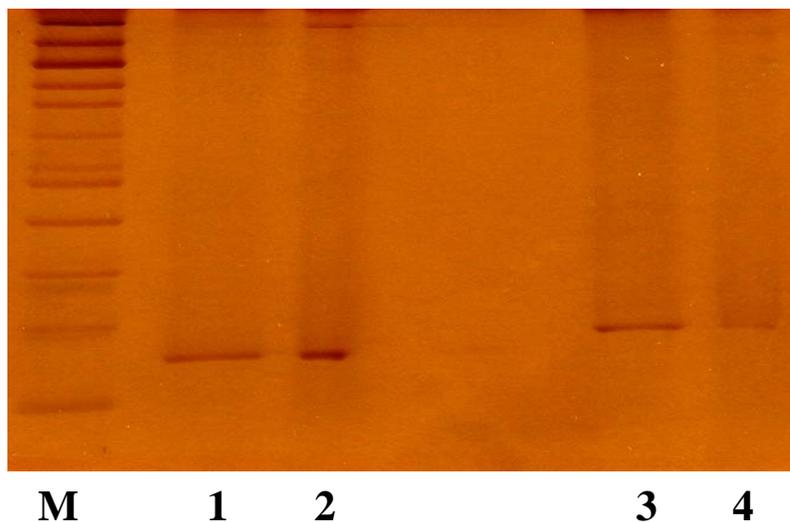
Figure 3.7   **SSCP analysis of human TTR exons**

SSCP analysis was performed to determine the shift in mobility due to conformational change of human TTR genes. The purified TTR exon amplified by PCR (100 ng) was denatured to a single-strand prior to analysis by native PAGE (20% resolving gel). The DNA bands were visualized by silver staining. (A) is TTR exon2 from ADHR control (lane 2-5) and ADHR (lane 1 and 6). (B), (C) is TTR exon4 from ADHR (lane3 in (B), lane4 in (C)) and ADHR control (lane 1-2, 4-7 in (B), lane 1-3, 5 in (C)).(D) is TTR exon1 from healthy control (lane1-3) and Down syndrome cases (lane 4). (E) is TTR exon1 and 4 from different source i.e. brain tissue (lane 2; TTR exon4 and lane 4; TTR exon1) compared with whole blood or lymphoblast (lane 1; TTR exon4 and lane 3; TTR exon1). M is the DNA marker (100 bp DNA ladder). Changes in mobility of the exons are indicated by broken circles.

4. Cloning of TTR exons for nucleotide sequencing

To obtain nucleotide sequences, the amplified TTR exons can be subjected to the sequencing directly. However, according to limit of the sequencing technique, sequences of the nucleotide at and flanking (~30-40 nucleotides away) the primer priming position can not be determined. Therefore, cloning of the amplified DNA into a vector and do sequencing of the DNA, while being an inserted form, is an effective way to obtain the whole sequence of the target DNA. In this thesis, nucleotide sequencing was carried out with TTR exons both as the PCR products and those inserted in a vector. For cloning, the PCR products were purified as described in section 2.8 and section 2.10 prior to insert into pGEM-T Easy vector at the *EcoR* I site(Figure 2.1, section 2.5). In vector, the DNA fragment was placed after the promoter of enzyme β-galactosidase (or Lac Z gene). The recombinant plasmid was then transformed into bacterial competent cell that prepared according to the method in section 2.7. The recombinant clones could be identified by blue/white screening using 5-bromo-4-chloro-3-indolyl- β-D-galactoside (X-gal) as the chromogenic substrate (Horwitz *et al*., 1964). The molecular mechanism of the blue/white screening is based on Lac operon of the *E. coli*. Insertion and expression of the TTR gene within the Lac Z gene lead to disruption of the hydrolytic activity of β-galactosidase. In the present of unligated vector, hydrolysis of the colorless substrate, X-gal, by the β-galactosidase causes the characteristic blue color in the colonies. While, insertion of TTR gene into the Lac Z region disrupts and loses ability of the *E. coli* in hydrolyzing the X-gal. Therefore, white colonies indicate gene insertion into the Lac Z region.. However, a bacterial colony that contains no vector, so that can not produce the β-galactosidase, also becomes white. However, these colonies can be suppressed by growing in the media supplemented with some antibiotic such as ampicillin.

Three to five white colonies were selected for plasmid isolation by the alkaline lysis in which the isolation is based on a unique property of plasmid that rapidly anneal following denaturation, allowing it to be separated from chromosome of the *E. coli*. The TTR exon1 that inserted in the plasmid is released and size can be checked after digesting the plasmid with *EcoR* I. It showed that sizes of the inserted TTR exon1 was 291 kb (Figure 3.9) as expected.

Base pairs
(bp)

1,517 ——

500 ——

100 ——

M   1   2   3   4
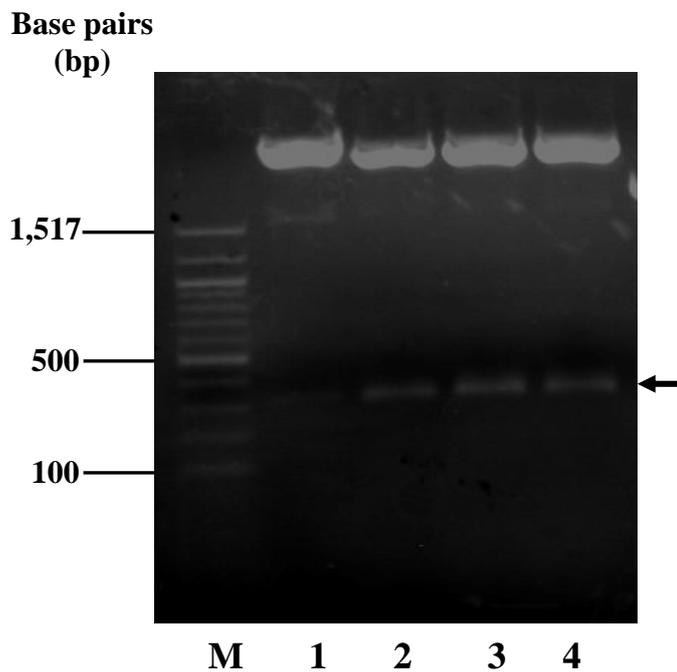
Figure 3.8   **Digestion of TTR DNA inserted plasmid by restriction endonuclease**

A recombinant vector (2.5 to 5.0 μg) containing TTR exon 1 was digested with *EcoR* I (10 units) prior the digestion reaction was analyzed on 1% agarose. M is the DNA marker (100 bp DNA ladder). Arrow indicates the DNA insert released out from the vector. 1 to 4 is an individual recombinant plasmid.

In contrast to the chain-termination developed by Sanger (Sanger *et al*., 1977), the chain terminators, i.e. dideoxynucleotides (ddATP, ddGTP, ddCTP, and ddTTP), rather than primers were labeled and used in the dye terminator sequencing. The major advantage of the dye terminator sequencing is the sequencing can be performed in a single reaction because the four dideoxynucleotide chain terminators are labeled with different fluorescent dyes at which fluorescing different wavelength. Together with high-throughout computer-controlled DNA sequence analyzer, the fluorescent signals generated from the incorporation of the dideoxynucleotides are collected and chromatogram of the sequence is created. Nucleotide sequence of the TTR fragments was determined by the dye terminator sequencing method as described in section 2.6. Nucleotide sequence of the abnormal TTR gene fragment primarily screened by SSCP analysis was aligned with the sequences of cDNA and the whole gene of human TTR deposited in GenBank (accession no. M11518 for TTR gene). The alignment showed a single base mutation occurred in the TTR intron1 rather than the exon1 region, at the nucleotide position 22 downstream of the 3′ end of the TTR exon1 (Figure 3.10). It is suggested to be a single nucleotide polymorphism (SNP), which C was substituted by G, of the TTR intron.

A

B

C
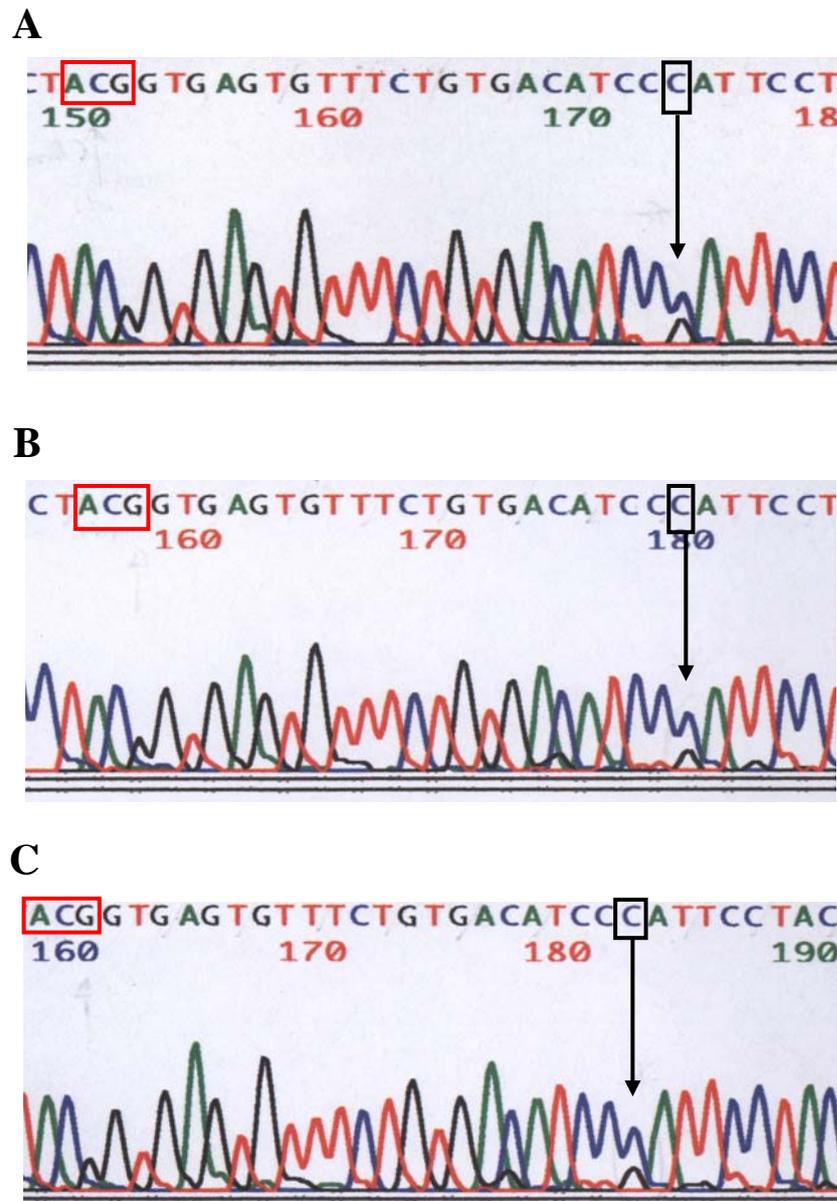
Figure 3.9 **Single nucleotide polymorphism of TTR intron**

The DNA fragments of TTR exon1 including partial parts of presegment and intron1 was amplified by PCR from genomic DNA of DS (A) and two normal persons (B and C) were determined for nucleotide sequences by dye terminator sequencing. Substitution of C by G was detected at position 22 of all these TTR intron1. ACG in red box are codon coding for the amino acid at 3′end of the TTR exon1. Substitution of C by G at position 22 of the TTR intron1 was shown in black box.

CHAPTER 4

CONCLUSIONS

1. DNA fragments of ApoE exon4, TTR exon1, exon2 and exon4 was amplified by PCR from 250 individual of Thai people showed single bands with the fragment size of 300, 291, 311, 258 bp, respectively.

2. Most common of ApoE genotype and ApoE allele was ε3/ε3 and ε3, respectively.

3. In this thesis, no ε2 homozygous that has been confirmed associate with longevity was not detected in all four Thai groups studied in this thesis, leading to a postulation that ε2 homozygous protect the development of dementia in only DS and elderly.

4. The frequency of the ε4 homozygous in the DS was three times higher than the normal, differs from the findings in DS from other ethnics. It is convincible that the Thai DS may develop AD faster than the DS from other ethnics.

5. The ε4/ε4 genotype in the ADHR was triple and the ε4 allele was double in frequency comparing to the control, suggesting high risk to develop dementia and AD in this Thai group.

6. By the SSCP screening, five of the DNA samples isolated from 173 individual people and the paraffin-embedded tissue showed abnormal in the electrophoresis mobility of the TTR exon1 or exon4 fragments. The nucleotide sequencing revealed that three of the five were the C→G substitution SNP in TTR intron1 at position 22 downstream to the 3′end of the TTR exon1.

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การศึกษาวิจัยที่ทำการทดลองในมนุษย์เรื่อง : เรื่อง "การเตรียม cDNA library และตรวจหา TTR variants ในคนไทย"

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