Association between Telomere Lengths and Cell-cycle Checkpoint Genes with Global Cognitive Function in the Hong Kong Chinese Older Community

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Role of Author

The thesis aims to evaluate genetic risk factors associated with cognitive impairment and Alzheimer's disease in Chinese older persons in Hong Kong. The clinical team of the Department of Psychiatry was responsible for subject recruitment, obtaining informed consent and clinical assessment in the clinical AD study. For the cell cycle gene and telomere length association studies with clinical AD, I was responsible for preparation of project proposal, DNA sample extraction, SNPs and telomere measurements and statistical analysis. For the healthy aging men study, the research team of Professor Woo of the Department of Medicine and Therapeutics was responsible for subject recruitment, cognitive assessment, blood sample taking and telomere length measurement, In this study, I was responsible for data analysis relevant for this thesis. I was also responsible for writing up the thesis.

ABBREVIATIONS

18FDG	18-fluorodeoxyglucose
AD	Alzheimer's Disease
ApoE2	Apolipoprotein E2
ApoE3	Apolipoprotein E3
ApoE4	Apolipoprotein E4
APP	Amyloid precursor protein
Aβ	Amyloid beta
CDK2AP1	Cyclin-dependent kinase 2-associated protein 1 gene
CDK5R1	Cyclin-dependent kinase 5 activator 1 gene
CDKN1A	Cyclin-dependent kinase inhibitor 1A gene
CDKN1B	Cyclin-dependent kinase inhibitor 1B gene
CDKN2A	Cyclin-dependent kinase inhibitor 2A gene
CKI	Cyclin-dependent kinase inhibitor
CNR2	Cannabinoid receptor 2
CNS	Central nervous system
CSF	Cerebrospinal fluid
DC	Dyskeratosis Congenita
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FDA	The U.S. Food and Drug Administration
GRIK	Glutamate receptor, ionotropic, Kainate 4
GSK3B	Glycogen synthase kinase 3 beta gene
HDAC	Histone deacetlylase
HIST1H3E	Histone cluster 1 H3e
IDE	Insulysin
IL-1	Interleukin-1
INK4	CDK inhibitor p16
LTD	Long-term synaptic depression

MAPT	microtubule-associated protein tau gene
MCI	Mild cognitive impairment
MDM2	Murine double minute 2 gene
MMSE	Mini-mental state examination
MRI	magnetic resonance imaging
NEP	Neprilysin
NFT	Neurofibrillary tangles
NMDAR	M-methyl-D-aspartate receptor
NPV	Negative predictive value
p53	Tumor protein p53 gene
PCNA	Proliferating cell nuclear antigen
PET	*Positron Emission Tomography*
PIB	[N-methyl-11C]2-[4'-(methylamino)-phenyl]6- hydroxybenzothiazole
PPV	Positive predictive value
pRB	Retinoplastoma protein
RB1	Retinoblastoma 1 gene
RNA	Ribonucleic acid
RT-PCR	Real-time polymerase chain reaction
SNP	Single nucleotide polymorphism
SNX2	Sorting nexin II
TNF-α	Tumor necrosis factor-alpha
TPDP1	Transcription factor Dp-1 gene
TRF	Terminal restriction fragment

ABSTRACT

Alzheimer's disease (AD) is the most common form of dementia. As the prevalence of AD increases with age, population aging will inevitably lead to an exponential increase in the proportion of older persons suffering from this disease. According to 2005 WHO estimate, 26.6 million people (approximately 0.55% of the general population) suffered from this disease. AD not only affects intellectual and functional abilities, it is also associated with significant neuropsychiatric disturbances. The pathogenesis of AD is characterized by widespread cerebral atrophy, abnormal deposition of amyloid plaques and tau protein in the central nervous system. While the classical histopathological features of AD are well recognized, exact physiological mechanisms that initiate the cascade of neural degeneration are still under active investigation.

Recent researches begin to unfold the physiological significance of telomere. A telomere is a repetitive region at the end of a chromosome. Basic functions of telomeres are involved with protection of the chromosome during replication and preventing chromosomal rearrangement or fusion. Abnormal telomere lengthening may be related to cancerous conditions. At a cellular level, telomere may also be related to aging and limitation in cell lifespan. In my study, I aimed to evaluate the

association between the lengths of telomere and global cognitive function in community dwelling Chinese older persons in Hong Kong. As the length of telomere is also determined by the turnover rates of cells, apart from association study of telomere lengths and cognitive function, I also tried to study the association of genes related to cell cycles and AD. Polymorphisms of ten cell-cycle checkpoint genes, i.e. RB1, CDKN1A, CDK5R1, CDK2AP1, CDKN2A, CDKN2C, MDM2, P53, GSK3B, TPND1 and CDKN1B genes, were chosen in my project.

The thesis comprised of three studies. The first study was an association study of cell cycle checkpoint gene single nucleotide polymorphisms (SNPs) with clinical diagnosis of AD. The second study was an association study of telomere lengths and clinical diagnosis of AD in a clinical sample of patients suffering from the disease. The third study was an association study of the telomere lengths and global cognitive status in a group of active community dwelling older men who participated in a healthy aging study

For the first association study of the cell cycle checkpoint genes and AD, sample was recruited from a prospective study of cognitive function and risk factors for development of AD. 701 elderly were clinically evaluated for diagnosis of AD by

psychiatrists. For this sample, genotyping of tagging SNPs of the 10 cell-cycle checkpoint genes were carried out by Restriction Fragment Length Polymorphism (RFLP) analysis. All tagging SNPs were selected from HapMap database and 5000bp upstream and downstream regions of each gene was also included.

As mentioned, the telomere length studies focused on ethically Chinese subjects recruited from two independent samples. The first clinical sample consisted of 411 older people and the other sample from healthy aging study, 976 community dwelling men were recruited. All subjects were assessed with the Cantonese version of the Mini-mental State Examination (CMMSE) for global cognitive function. Genomic DNA of the subjects was extracted from the peripheral whole blood sample. Lengths of the telomere were measured with Quantitative Real-Time PCR and the Ct ratio of the telomere and a control gene (36B4) of each sample was compared with the standard curve constructed with 4 selected sample's telomere lengths measured previously by Southern blotting.

For the results, the association study with cell cycle checkpoint genes, there was no SNPs found to be associated with diagnosis of clinical AD. We also found out that telomere length was associated with age in both two healthy aging men and clinical

samples. There was no association between education and telomere lengths. For subjects in the healthy aging study, participants with CMMSE scores fell into the lowest 25% were found to have shorter telomere lengths. Similar result was found in the clinical AD sample.

In the study, telomere lengths were negatively associated with age. As the telomere will be shortened for each cell cycle, this finding correlated with physiological function at a cellular level. Statistical analysis also showed that shorter telomere lengths were found in subjects with poorer cognitive function. However, as age is a major determinant for cognitive impairments, further studies are recommended to evaluate the interaction effects of age in this association. Telomere shortening will cause cell senescence, and may be associated with faster neuronal degeneration, thus affecting cognitive function. Further studies should be conducted to examine its usefulness as an adjuvant biomarker for risk stratification of AD intervention trials.

摘要

阿氏癡呆症 (Alzheimer's disease, AD) 是最常見的癡呆症。由於阿氏癡呆症的廣泛性會隨著年紀而增加，所以在人口老化的社會裡受到阿氏癡呆症影響的老人人數會以很快的速度增加。根據 2005 年世界衛生組織估計當時有大約二千六百萬人患上阿氏癡呆症，估當時人口大約 0.55%. 阿氏癡呆症不單會影響患者的智能和日常行爲，而且也會在精神上造成極大困擾。阿氏癡呆症在病理上的特徵包括腦細胞死亡，乙型類澱粉樣蛋白的沉澱及神經元纖維纏結。雖然在病理組織上阿氏癡呆症的特點是十分明顯，但是在致病的原理上仍然是未有確實答案。

另一方面，近年來端粒對生理上的重要性受到重視。端粒是在染色体上末端一段擁有重覆排序的結構，而它可以避免細胞受末端複製問題的影響和跟壽命有關。不過不尋常的端粒可能會與癌症有關。我的研究主要集中尋找端粒長短與在港中國老人認知能力上的關係。由於端粒長短跟細胞復製次數有關，所以我亦會嘗試研究阿氏癡呆痴跟細胞週期的關係，當中會集中在十個負責確保細胞週期運行準確性的基因上的異變。

研究包括三方面，第一方面是與細胞週期有關的基因的單核苷酸多態性與阿氏癡呆症的關係。第二是發掘在阿氏癡呆症患者中端粒長度與癡呆症的關係。第三

是在健康的老人人口中端粒長度跟認知能力的影響。

結果顯示，在與細胞週期有關的基因的單核苷酸多態性與阿氏癡呆症的關係研究中，在七百零一位研究對象中，阿氏癡呆症未有發現跟十個被選中跟細胞週期有關的基因異變有特別關連。而在四百一十一位臨床和九百七十六位健康的老年研究對象中，我們發現端粒長度在認知性測試(MMSE)較差的對象中顯示出關連，但是由於年齡是認知衰退最主要的因素，所以仍需要更多的實驗研究去發掘年齡、認知衰退和端粒長短的關係。我們亦都發現端粒長短和年齡有反向性的關係，但在教育程度上就沒有發現關連。

總括而言，今次研究成功發現端粒長短在認知能力較差的人口中有關係，但對於能否應用於臨床上就需要更多的研究。

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CHAPTER 1. Introduction

1.1 Alzheimer's Disease (AD)

1.1.1 Prevalence

AD was named after German psychiatrist Alois Alzheimer for his first report of the disease in 1906. It is the most common form of dementia (1). According to the Alzheimer's Association data, AD accounts for around 50% to 80% of all dementia (2).

From a recent Delphi consensus study (3), the prevalence of the disease in 14 world regions was estimated. The study estimated that there are around 24 million people suffered from dementia in 2005, and the population was forecasted to double reach 42 million in 2020 and 81 million in 2040.

The study also indicated that the proportional increase in persons with dementia will be much higher for the developing countries than that in developed countries. At present, 60% of the total population of people with dementia is from the developing region, the figure will increase to 71% in 2040.

In the 2009 World Alzheimer Report prepared by Alzheimer's Disease International (ADI) (4), it is estimated that there is more than 35 million patients suffering from AD in 2010. The AD population is predicted to be doubled to

around 65.7 million in 2030 and will further explode to approximately 115.4 million in 2050.

According to the information of Hong Kong Population Projections 2007-2036 (5), the proportion of elderly over 65 years old will raise from 12% in 2006 to 18% in 2021 and further increase to 26% in 2036. An earlier study estimated that the prevalence of dementia was around 6.1 and that of AD was approximately 4% (6). In a more recent study carried out in 2007 with sample size of 6100 (7), 8.9% of elderly aged 70 or above suffered from mild dementia and 73.5% satisfied diagnostic criteria for possible AD. A rise in prevalence of AD may be explained by increased life expectancy, relatively low literacy attainment and midlife cerebrovascular risk factors (8).

1.1.2 Clinical Features

The spectrum of cognitive abilities in late life is wide. Older people may thrive with successful aging having no significant cognitive and functional loss as age advances (9). They may suffer from physiological aging when attentional ability or processing speed is reduced, but independent functioning is still preserved. Clinical dementia represents the worse end when people will pass through stages of progressive cognitive and functional deterioration till total dependency. There

is also an at-risk stage between normal ageing and clinical dementia. The stage may be represented by different terms, the most well known is mild cognitive impairment (MCI). For MCI, the predominant symptom is memory loss and the individual usually has a subjective sense of his or her own problem. Cognitive function may be mildly impaired, but people with MCI do not satisfy criteria for clinical dementia. Their everyday functioning abilities are still maintained at a relatively independent level. As an at-risk state, an ADNI (Alzheimer's Disease Neuroimaging Initiative) cohort study in 2009, it was suggested that about 10% to 15% of people with MCI will progress to probable AD each year using MRI scans. It is also interesting to note that some subjects with MCI are able to maintain at their level without converting to dementia with time.

Clinical AD may be divided into mild, moderate and severe stages according to the severity of cognitive and functional impairment (10, 11). In the mild stage, recent memory loss is prominent with remote memory slightly better preserved. Patients may exhibit difficulties in language expression and understanding. Apart from that, difficulties in motor coordination, neuropsychiatric symptoms and personality changes may also manifest. Deficits in everyday functioning are present, but basic self care is usually maintained. The mild stage of AD may last for a few years before progressing to the moderate stage.

In the moderate stage, patients start to loss their ability for independent living and find it difficult to perform basic self care. Different domains of Cognitive function are impaired, long term and short term memory problems are obvious. Prominent neuropsychiatric symptoms occur in patients with moderate AD. Common symptoms include perceptual abnormality, mood and activity disturbances as well as vegetative symptoms. Persons with moderate AD will need to be taken care of by caregivers. The duration of illness is highly variable, and the moderate stage may last for many years before further progression.

The moderate stage will progress to the late (severe) stage after 2 to 10 years. In this stage, the patient has lost the independence and daily living has to be dependent on caregivers. Language expression and understanding deteriorated. People with AD may be unable to communicate in full sentence. Neuropsychiatric and behavioral disturbances are prominent and less organized. Patient will not able to finish simple tasks due to motor coordination incapacity. Eventually, persons suffering from advanced AD will be totally dependent and maybe bedridden.

1.1.3 Diagnosis

Most elderly with AD are not diagnosed early. In order to raise public awareness

of AD symptoms, the Alzheimer's Association published a list of 10 signs of Alzheimer's (12), i.e. (1) memory loss that disrupts daily life, (2) challenges in planning or solving problems, (3) difficulty completing familiar tasks at home, at work or at leisure, (4) confusion with time or place, (5) trouble understanding visual image and spatial relationships, (6) new problems with words in speaking or writing, (7) misplacing things and losing the ability to retrace steps, (8) decreased or poor judgment, (9) withdrawal from work or social activities and (10) changes in mood and personality.

The standard diagnostic criteria of AD are the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria established in 1984 (13), and updated in 2007 (14). The criteria include emphasized on progressive and global cognitive impairment, supported by neuropsychological testing and neuroimaging assessment for AD diagnosis, and had been validated with postmortem histological correlation.

1.1.4 Screening tests

The mini-mental state examination (MMSE) is the most widely used clinical screening tool for AD (15). MMSE screened for different areas of cognitive

function, i.e. orientation, registration, working memory, episodic memory, language, complex commands and visuospatial task. The total score is 30 and the usual cutoff score used to distinguish normal and dementia is 24 in the Caucasian population (16). For the Hong Kong Chinese community, owing to variations in educational and cultural background, a different cutoff level was adopted (6). The cutoff point of 18 was set for non-educated elderly, twenty for those received less than 2 years of education and cutoff 22 was used if subjects had longer than 2 years of education.

1.1.5 Histopathology

There are 2 major hallmarks identified in the brains of persons with AD, the presence of amyloid plaques and neurofibrillary tangles.

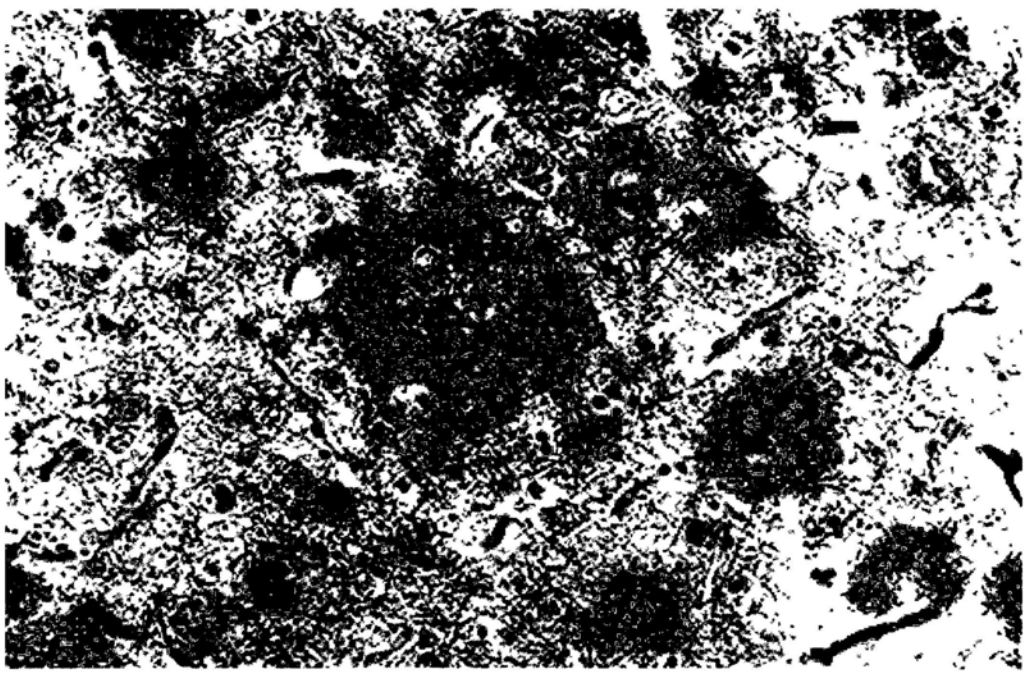

Figure 1.2 A microscopic picture of silver stained plaques of Alzheimer's disease.

(Photo adapted from http://library.med.utah.edu/WebPath/CNSHTML/

CNS090.html)

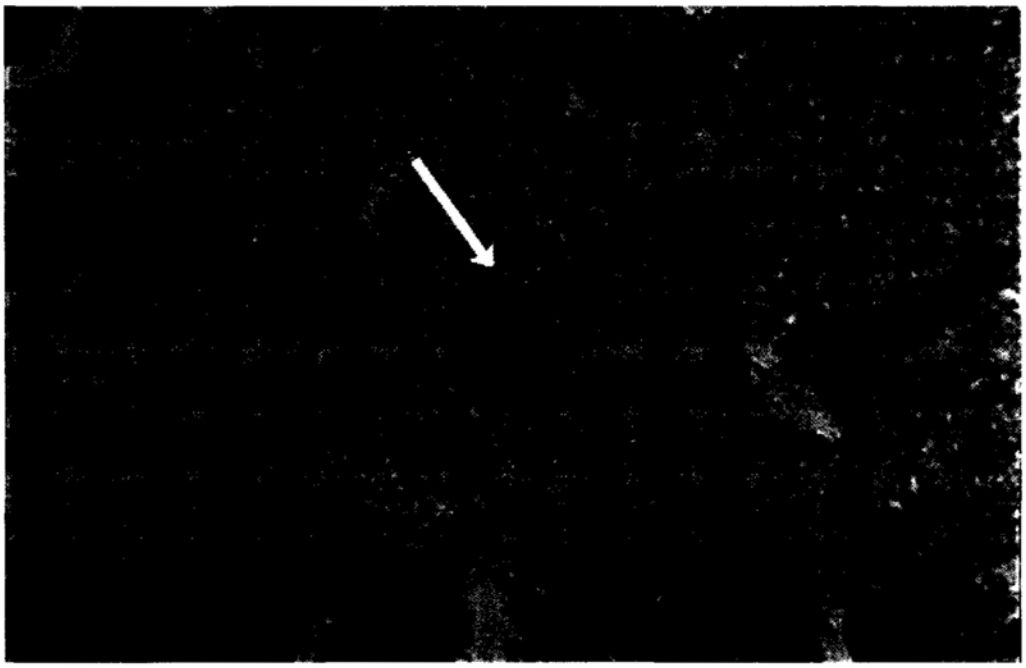

Figure 1.3 A microscopic picture of neurofibrillary tangle of Alzheimer's disease. The tangle is stained as long pink filaments in the cytoplasm.

(Photo adapted from: http://library.med.utah.edu/WebPath/CNSHTML

/CNS094.html)

1.1.6 Pathology

Amyloid plaques are found between neurons, and are mainly constitutes of amyloid beta peptides build up with 39 to 42 amino acids (Aβ) (19-22). Amyloid beta is formed by cleavage of amyloid precursor protein (APP). The gene of

amyloid precursor protein (APP) is located in the chromosome 21. APP is a transmembrane protein and commonly expressed in the brain. APP is suspected to function as cell receptor, adhesion, formation and repair of synapses. Under normal situation, APP cleaved by α -secretase. Sequential action by β-secretase will produce soluble APP derivatives which are apparently harmless to neurons. However, if the APP is cut by β - and γ-secretases, an insoluble product will be resulted and the derivative is amyloid beta (Aβ42). The most 2 common forms of amyloid beta are Aβ40 and Aβ42. Aβ42 is more hydrophobic and fibrillogenic, and is commonly found in amyloid plaques of AD brain. Amyloid plaques are suggested to be the fundamental pathological mechanisms leading to AD. However, the story is complicated by subsequent findings that correlations between intensity of AB deposition and clinical severity are not direct (23-25).

While amyloid plaques locate between cells, neurofibrillary tangles (NFT) are found within neurons. The tau proteins are formed by alternative splicing of the gene microtubule-associated protein tau (MAPT) located in chromosome 17. There are total six isoforms produced by the splicing of the MAPT in human brain. Each isoform has its own pattern and number of binding domains. Three isoforms have three binding domains while the other three, including tau, have four binding domains. Tau proteins are abundantly expressed in CNS neurons as

soluble proteins. Its normal function is to stabilize microtubules by interacting with tubulin. However, in AD brain, it is found that tau proteins are abnormally hyperphosphorylated and resulted in insoluble aggregation of proteins. Aggregation of tau proteins is likely to be pathogenic as some studies suggest that neurodegeneration occur in the presence of NFT even there is no amyloid plaque found (26-31).

1.1.7 Other Related Mechanisms

i. Apolipoprotein E4 (ApoE4)

The main function of apolipoproteins is to transport dietary fats, fat-soluble vitamins and cholesterol through bloodstream by binding hydrophobic lipid to form lipoproteins (32-36). Apolipoprotein also acts as coenzymes and ligands of lipoprotein receptors. There are six classes of apolipoproteins, i.e. A, B, C, D, E and H.

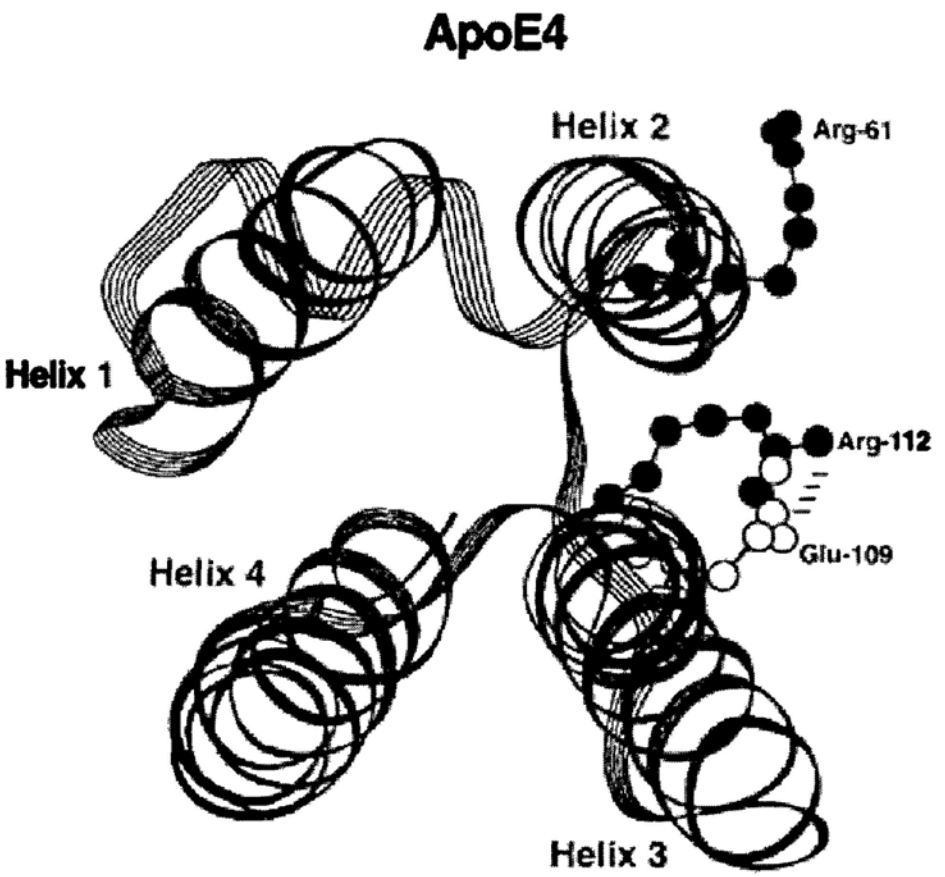

Figure1.4 3-D structure of apolipoprotein E4 (37)

The ApoE gene is mapped in the chromosome 19 with 3597 base pairs. ApoE4 is one of the 3 isoforms of apolipoprotein (Apo), the other two are E2 and E3.

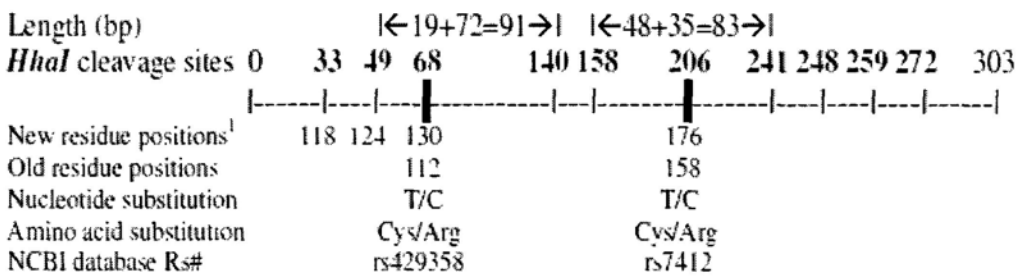

Figure 1.5 The diagram shows different Hhal restriction sites within the ApoE amplicon and different allele combinations define the class of ApoE (38).

The ApoE2 consists of allele T at the SNP rs429358 and T for the SNP rs7412 whilst the haplotype of ApoE3 is T and C for rs429358 and rs7412 respectively. For ApoE4, the substitution polymorphism at the site rs429358 and rs7412 are allele C (38).

The most frequent allele in human is ApoE3 and the least frequent one is ApoE2. However, relative frequencies of alleles in different parts of the world varied. In Papua New Guinea, the most common allele is ApoE4 and the frequencies of ApoE4 are relatively high in Africa while it is relatively low in Asia (39,40).

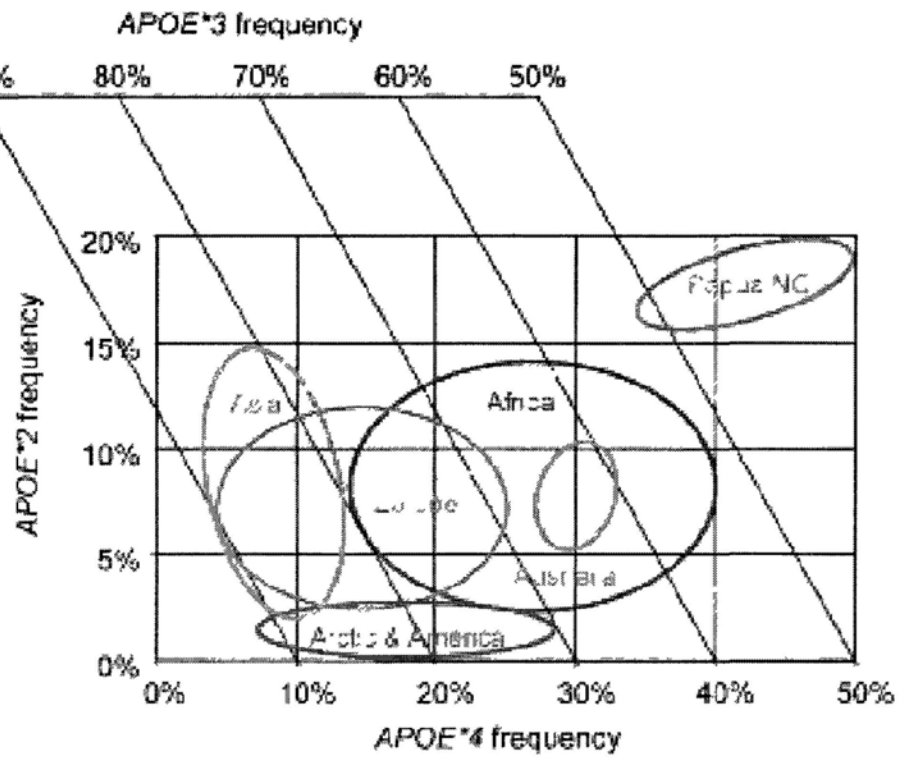

Figure 1.6 Allelic frequencies of ApoE2, ApoE3 and ApoE4 in different parts of the world (40).

In 1995, a seminal paper by Roses (38, 41-43) showed that ApoE4 could greatly increase the risk for late-onset AD. ApoE is the predominant apolipoprotien in the brain. It functions as a transporter of lipids in the brain and affects the rate of beta-amyloid protein (Aß) degradation (44). ApoE may interact with Aß and transfer Aß into brain neuronal cells (45). The ability of ApoE to remove Aß peptides depends on its lipidation status and type of isoforms (46). Animal model studies (47-49) demonstrated that APP transgenic mice (50-53), when expressed with different lipidated ApoE isoform, i.e. ApoE2, ApoE3 and ApoE, showed different degrees of Aß deposition inside their brain cells. Mice with the isoform ApoE4 expressed had largest amount of Aß accumulated, while mice with ApoE2 deposited least (54-56). The exact mechanism was unknown but it was believed that the variation of isoform-dependent Aß accumulation was correlated with the different affinity of lipidated ApoE to Aß, and ApoE4 was the less effective isoform to remove Aß from the brain. One of the hypothetic mechanisms was that apolipoproteins are highly expressed in astrocytes and microglia of brain (58,59), and ApoE lowered the level of extracellular soluble Aß by transporting Aß inside the cells and degraded the peptides proteolytically by two main enzymes, i.e. neprilysin (NEP) and insulysin (IDE) (60-62). As ApoE4 binds to Aß least effectively, they remove less amount of extracellular Aß thus favoring AD development.

The age of onset AD appeared to be associated with different ApoE alleles in a gene-dosage manner. People with ApoE4/E4 genotype were reported to have an increased opportunity to develop AD when compared with normal. ApoE2 is considered as protective to reduce AD development risk. (38).

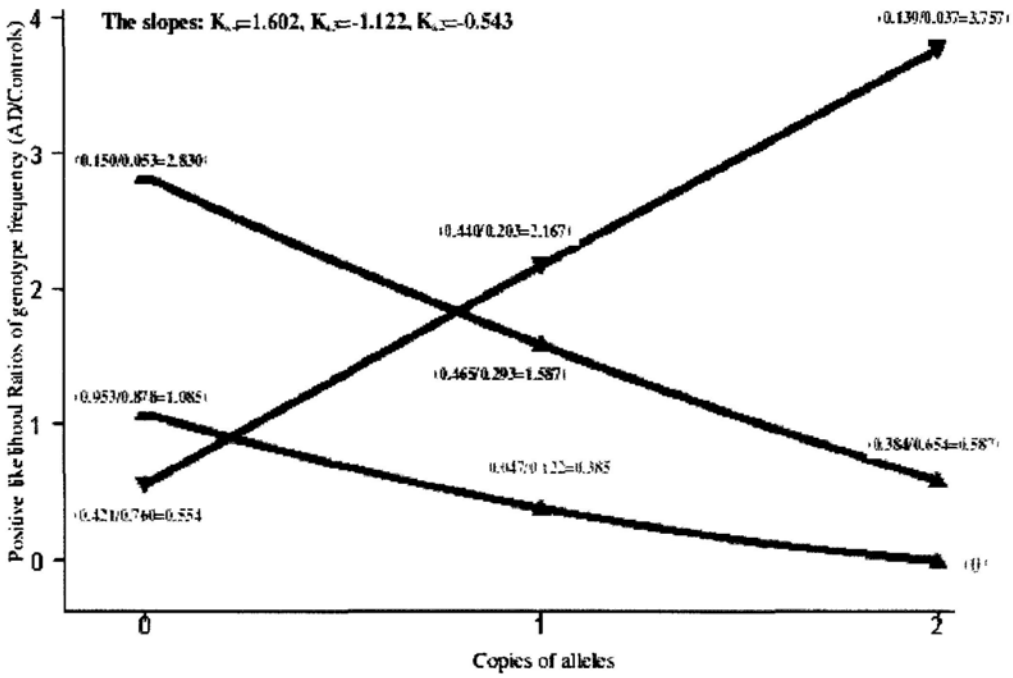

Figure 1.7 Gene-dosage effects of ApoE allele to the risk of onset AD (38)

Although ApoE4 is highly co-related with onset of AD, it is not the determining factor of the disease. Some people carrying homozygous ApoE4 alleles do not develop AD, and only two-third of AD patients contains ApoE4 allele in their gene. Some others factors are also contributory to the disease progression.

ii. Amyloid protein

Although the appearance of amyloid plaque in brain is one of the hallmarks of AD, the relationship between Aß and the initiation of AD is not cleared. However, it is believed that the plaque is the outcome of the disease progression, but soluble form of amyloid protein may also play a role in AD As it had been suggested that oligomers of Aß are neurotoxic and affect synaptic function.

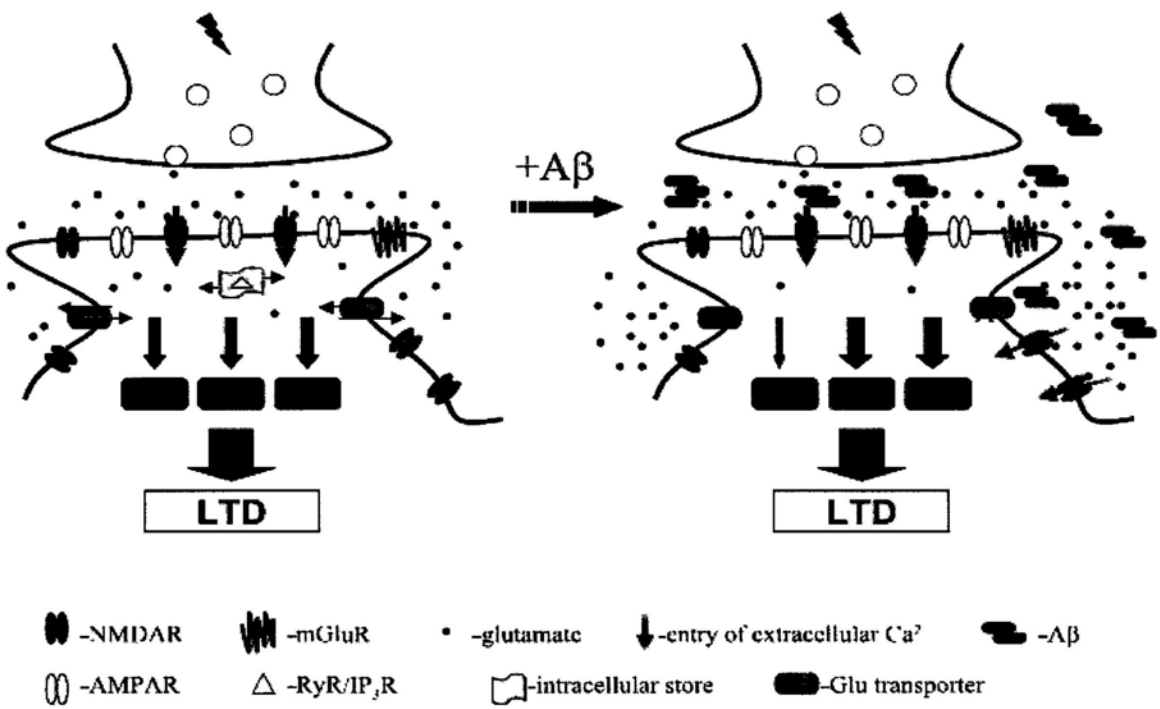

Figure 1.8 Signaling pathways of conventional long-term synaptic depression (LTD) and Aß-mediated LTD (63).

Conventional long-term synaptic depression is depended on calcium balance inside and outside the brain cell. M-methyl-D-aspartate receptor (NMDAR) mediates extracellular calcium influx and release of intracellular calcium.

However when there are soluble Aß presented, it will stimulate the inflow of

However when there are soluble Aß presented, it will stimulate the inflow of calcium into the cell as Aß can induce calpain-mediated cleavage and inactivation of ion channels and also inhibit the Flu-transporters which lead to decrease in glutamate uptake. The high level of intracellular calcium and hold in glutamate transportation may contribute to the induction of LTD (63-64).

iii. Tauopathies

Tauopathies are a group of neurodegenerative diseases caused by tau protein aggregation and AD is one of the examples (65-68). The aggregation of tau proteins, which lead to structural formation called neurofibrillary tangles (NFT), and is believed as a consequence of cytotoxicity effect of Aß.

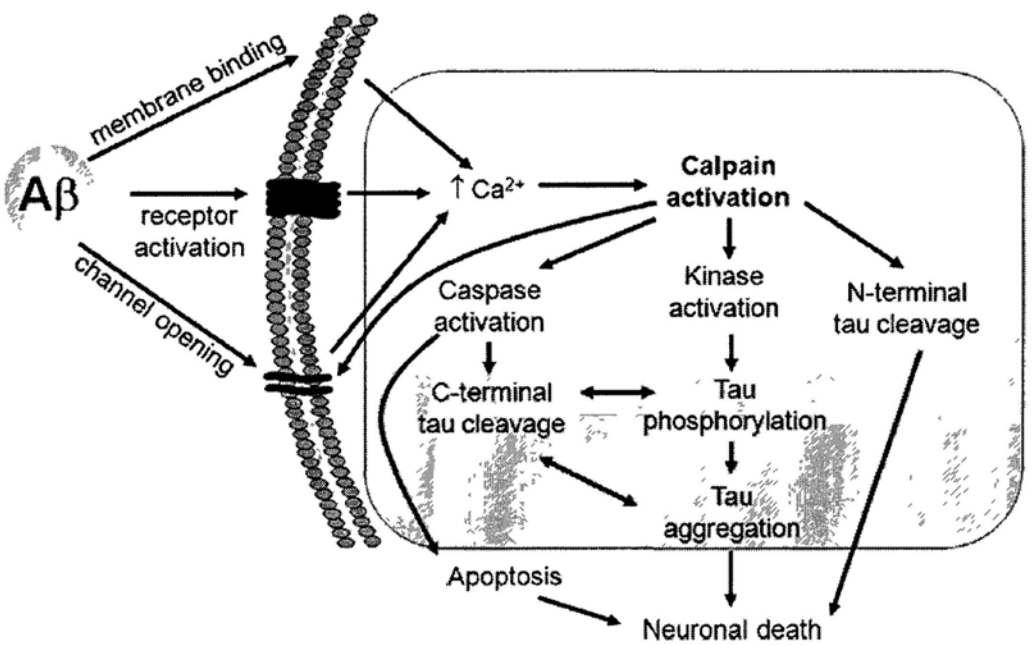

Figure 1.9 The Aß-induced neurodengenerative pathway including downstream

The presence of extracellular soluble Aß proteins will result in accumulation of calcium ions inside the neuron. High calcium level activates calpain and trigger downstream pathways which lead to cell death. The GSK3 and cdk5 are kinases and phosphorylated the tau proteins. Hyperphosphorylation of tau proteins lead to tau aggregation and neuronal death. The binding of tau protein to microtubules depends on well-regulated balance of phosphorylation status of tau proteins. This regulation is based on co-operated functions of kinases and phosphatases (69).

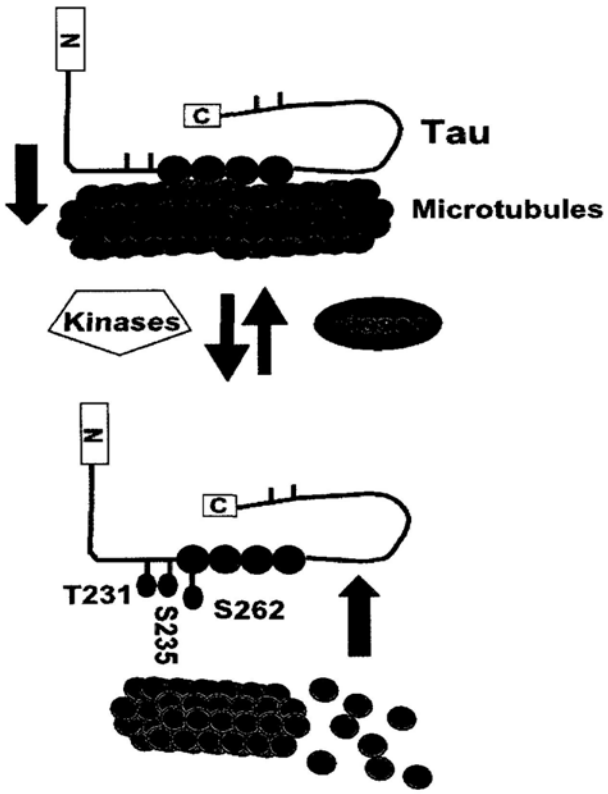

Figure 1.10 Diagram shows the phosphorylation status of tau protein and binding of tau to microtubules (69).

The highly regulated phosphorylation status is critical to the normal function of neuron because it controls the neuritis outgrowth. When there is high intracellular

neuron because it controls the neuritis outgrowth. When there is high intracellular calcium concentration, the activation of calpain will lead to activation of tau kinases and suppress the action of phosphatases. As shown in the above diagram, phosphorylation of tau at Ser262 and Thr231/Ser235 will trigger the detachment of the protein from microtubules. The free tau will be more susceptible to aggregation and formation of tangles.

Apart from the activation of kinases by calpain, it may also lead to activation of caspases, especially caspase-3. Caspases can truncate the amino or carboxyl terminals of tau protein. The removal of the terminal will greatly increase the risk of tau self-assembly as both terminals have inhibitory natures for aggregation (70-71).

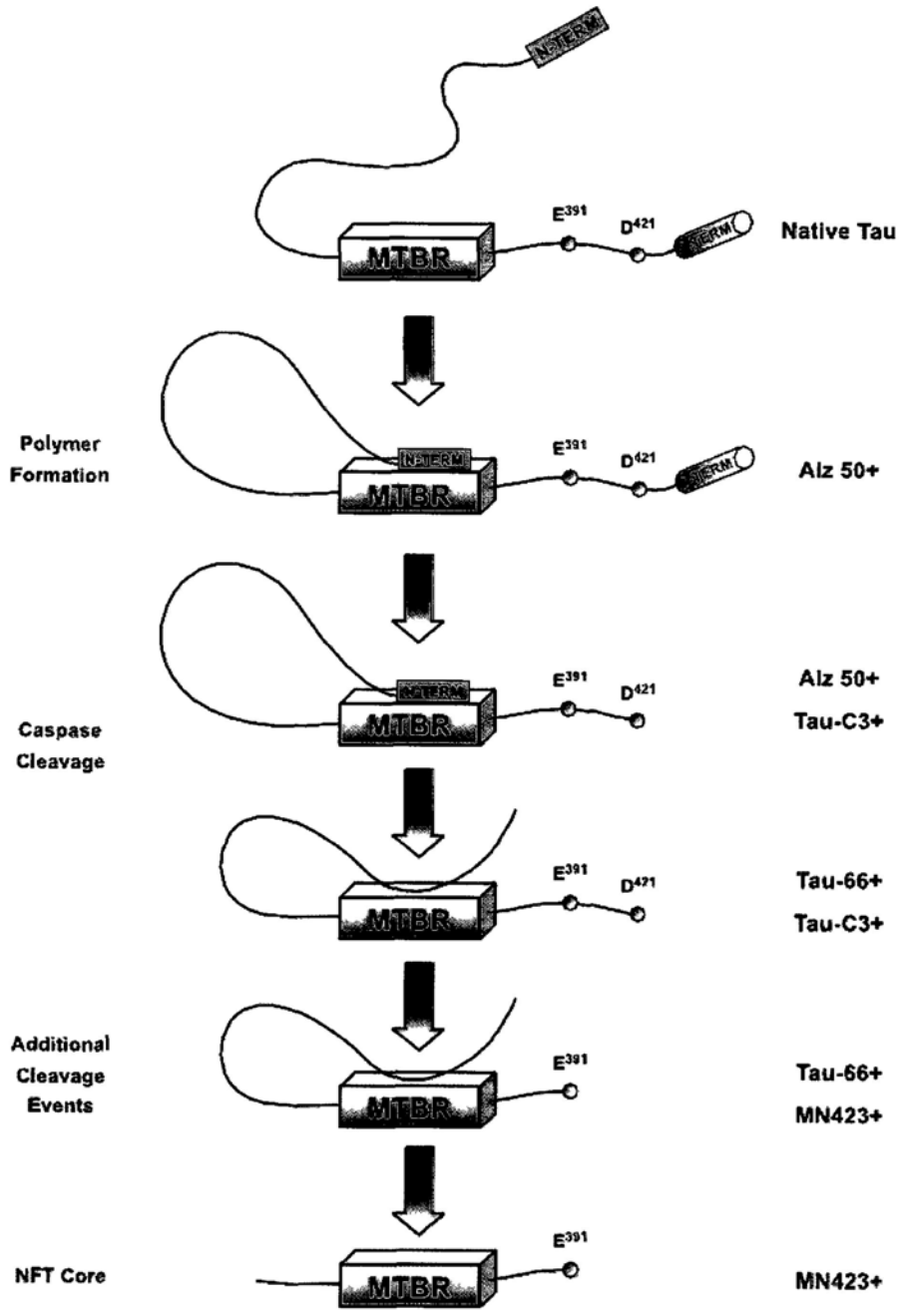

Figure 1.11 Different steps of removal of terminals of tau proteins and formation of NFT. Caspase cleaves the amino terminal of tau (shown by shifting detection by monoclonal antibodies Alz 50+ to Tau 66+) and subsequently the carboxyl terminal is removed (detected by antibody MN423+) (71).

iv. Inflammation

Inflammation is not recognized as the cause of AD but it may contribute to the disease progression. Chronic inflammation causes serious harm due to prolonged exposure to cytotoxic inflammatory molecules. The inflammatory hypothesis of AD builds on the observations that there are immune-related antigens and proteins co-localize with amyloid-beta (Aß) and tau proteins.

For immune response to be provoked, some molecules must be presented as foreign source to irritate body's self-defense mechanism. Aß is suggested to play the role of initiating immune response. The microglia in brain will be activated and try to clear the amyloid proteins and this response is an innate immune response. In normal peripheral tissue, after primary innate immune response, secondary adaptive immune response starts to clear any antigens. However, in AD brains, it is found out that there is impairment to activate microglial Toll-like receptor and adaptive immune response is not activated. As a result of that, the inflammation will be chronic and lead to release of cytokines, chemokines, reactive oxidative molecules and proteases which are toxic to neuronal cells and causing neurodegeneration (72-76).

The important role of immune response was shown in some studies that people with asymptomatic AD were found to have lower inflammatory activities (74, 76).

Also, some studies on people with rheumatoid arthritis with chronic anti-inflammatory prescription exhibited lower risks for AD (73, 77-78).

Some polymorphisms in genes related to inflammatory pathway had been reported as associated with the onset of AD. For example, genetic polymorphisms in Interleukin-1 (IL-1) (79) and tumor necrosis factor-alpha (TNF-α) (80) were associated with microglial activation in AD.

v. Cell Cycle and Regulation

Neurons are generally recognized as terminally differentiated and rest in the G0 phase cell cycle. Recent experiments suggested that neurons re-entered cell cycles, as many related proteins such as cyclin B, cyclin D, cyclin E, cdk4, Ki67, p16 and p106, which are normally only expressed in actively dividing cells, are found. Dysfunction of mitotic signaling pathway and abnormal cell cycle re-entry may be one factor contributing to the neurodegeneration. A cell cycle can be divided into 4 steps, i.e. G1, S, G2 and M phases. Thereis also a quiescent G0 for terminally differentiated cells like neurons (81-84).

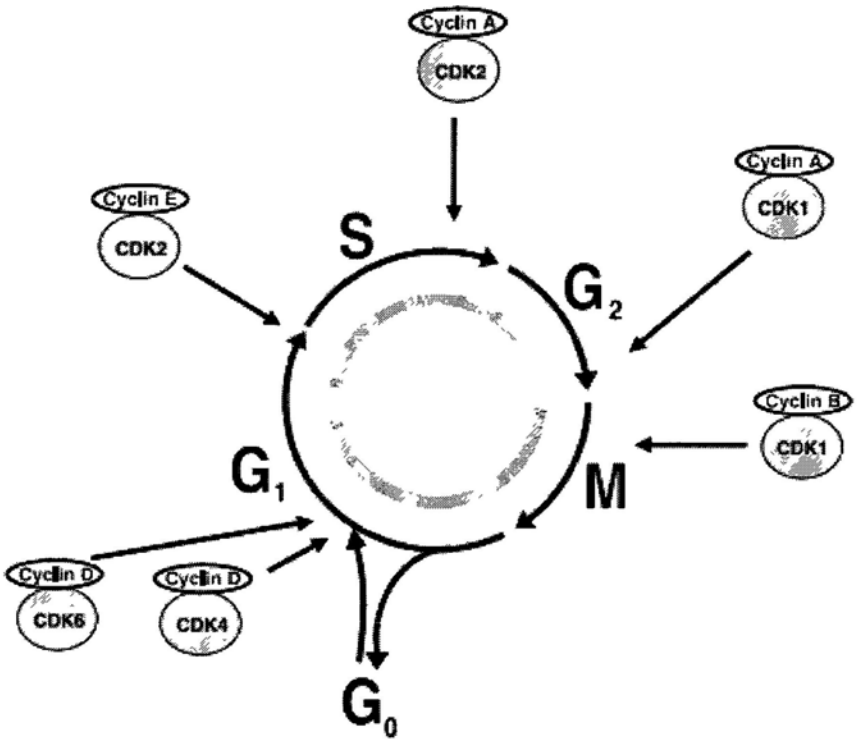

Figure 1.12 The 4 stages of cell cycle with the resting G0 phase. Also regulatory cyclin-CDK complexes for each stage are indicated (81).

The first stage of cell cycle is G1. During the G1 phase, the size of cell is enlarged. Organelles are replicated and many enzymes required for S phase are synthesized. No chromosomes are replicated yet. After lasting for around 9 hours in G1 phase, the cell will proceed to S phase. S phase is known as the synthesis phase as DNA replication occurs. Double helical DNA of chromosome will be opened and polymerase will produce a new polynucleotide strand from its complementary DNA strand. At the end of S phase, there will be 2 set of chromosome pairs (4n) in the cell. It is the rate-limiting step of cell cycle as post-DNA replication validating mechanism is also activated in S phase.

The third step in the cell cycle is G2 phase and it is another step for protein synthesis. Microtubules that will be required to chromosomes separation are produced. After that, the cells enter the final phase of replication, i.e. M phase. The two duplicated chromosome pairs (4n) separated into two daughter cells. It is followed immediately by cytokinesis, i.e. cytoplasm of parent cell containing previously synthesis organelles is equally separated into 2 daughter cells which containing identical genetic information.

a. Cell Cycle Checkpoints

There are 3 cell cycle checkpoints- G1, G2 and metaphase checkpoints (85-91). Checkpoint functions to prevent cell cycle progression from one phase to the following , before all events in the phase before checkpoint are all properly finished. DNA damage and other stress can trigger the activation of these checkpoints to hold the cell replication or may lead to apoptosis.

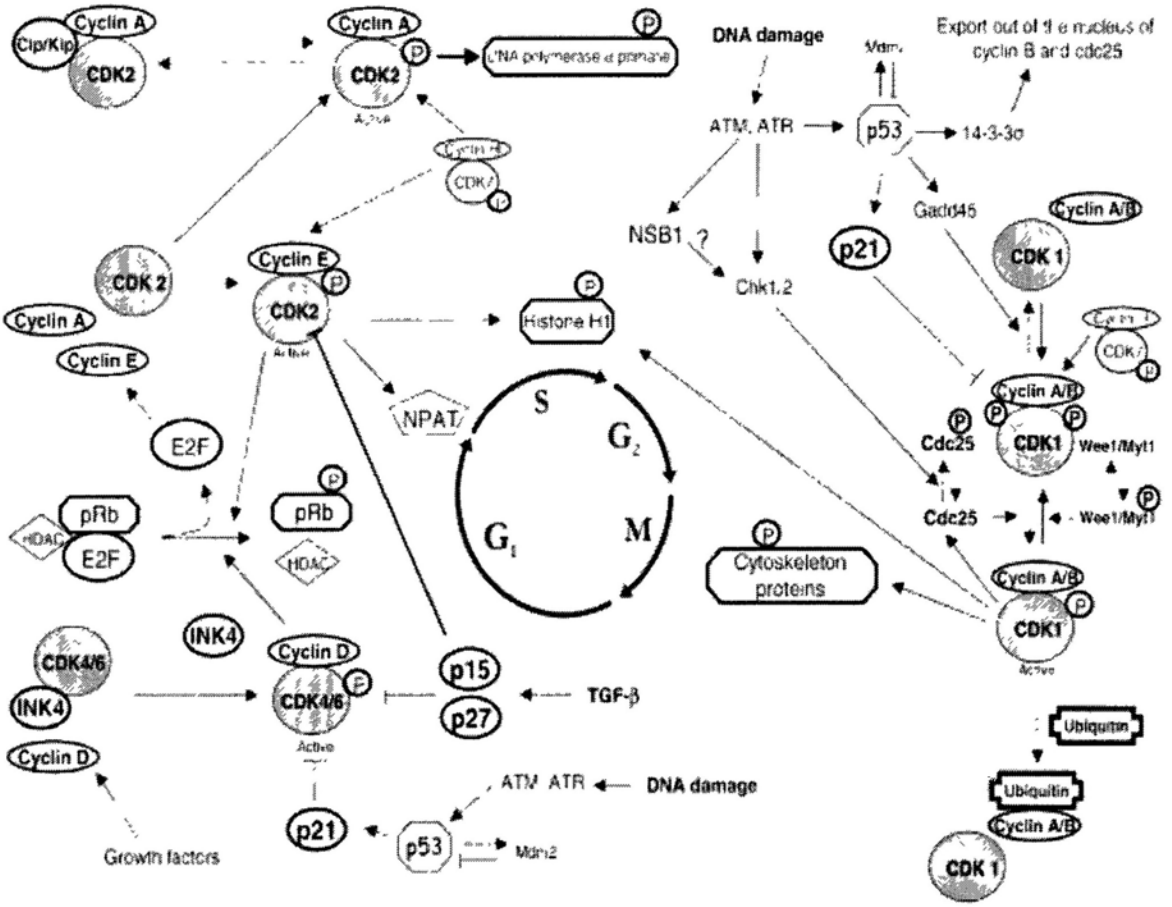

Figure 1.13 Detailed overview of cell cycle regulation with related pathways (81).

1. G1 Checkpoint

The G1 checkpoint is presented at the end of G1 phase before the start of S phase. This checkpoint will determine the "fate" of the cell to whether passing the cell to S phase for chromosomal replication or still in a resting state called G0, such as the liver or neuronal cells. The action of G1 checkpoint is mainly controlled by the protein CDK inhibitor p16 (INK4). INK4 protein can bind to CDK4/6 in order to prevent it from interacting with cyclin D. As cyclin D is necessary for the cell to

transit to S phase, the activation of INK4 will hold the cell at the checkpoint. However, if the cell is ready to replicate DNA, INK4 will detach from CDk4/6 and cyclin D complex and lead to sequential phosphorylation of retinoplastoma protein (pRb). Transcription factor E2F is released and cyclin E will be expressed which allow cell cycle transition from G1 to S phase.

2. G2 Checkpoint

This checkpoint is located between the end of G2 and beginning of M phase. Similar to the important role of INK4 in G1 checkpoint, a phosphatase Cdc25 plays the central part of G2 checkpoint. Cdc25 will phosphorylate the complex of CDK1 and cyclin A/B so the complex promotes the cell to enter M phase.

3. Metaphase Checkpoint

This checkpoint is presented within the M phase and the main function is to make sure all chromosomes are properly aligned in the middle of the cell before entering to anaphase, which separates duplicated chromosomes into 2 daughter cells.

vi. Telomere

a. Discovery of Telomere

Before the discovery of telomere, scientists had considered that normal cells were able to replicate indefinitely with their successful cultivation of many immortal cancerous cell lines like L929 and HeLa etc. Ageing was as a matter largely mediated by exterior factors but not those inside the cell (92-94).

The preliminary findings were, however, not replicable. Normal cells have limited replicative capacity. It was unaffected even after the cell was kept frozen for many years (95). When human fibroblast cells are put under studies, the limit of cell division was approximately 50 times. As a result, it was believed that there should be an intrinsic "clock" that controls the replicative capacity of the cell (92).

In 1938, Muller and McClintock discovered that a special region at the end of chromosome and coined this region as telomere. However the physiological function of telomere was not clear at that time. The association of telomere with DNA replication was discovered in 1972. Olovnikov unraveled the mystery of missing DNA upstream product in the lagging polynucleotide chain during mitosis, and recognized that the repeating unit at the end of the chromosome might act as a buffering region. The length of the telomere limited the number of replication that a cell may achieve. In 1978, Blackburn and Gall discovered the repeated sequence TTGGGG was located at the protozoan chromosomal end, while the

telomere repeats TTAGGG of human was discovered in 1988 (96).

b.Function of Telomere

Human DNA is made up of two polynucleotide chains, built up by joining deoxyriboses through covalent phosphodiester bonding. The two chains are running in opposite direction and form a double helical spiral structure.

During cell division, the double-stranded DNA will be separated by helicase to form an opening named "replication folk". DNA polymerase will start replicating the polynucleotide chain in a 5' to 3' direction. As a result, the leading polynucleotide chain (3' to 5' direction) will be opened up for the working polymerase to replicate nucleotides along the strand. When the polymerase encounters with the lagging chain (i.e. 5' to 3' direction), it is impossible to replicate the lagging polynucleotide strand in a simple head-to-tail manner. In order to solve the problem, our body uses temporary short RNA strands as primers to adhere to the DNA sequence located before the replication starting point and act as initiation sites so the DNA polymerase can start replicating the sequences from right behind the RNA primer to the head of downstream replicated DNA strand. The RNA primer will then be degraded and the gap will be filled by DNA and DNA ligase seal the strands to form a long intact DNA strand

(98).

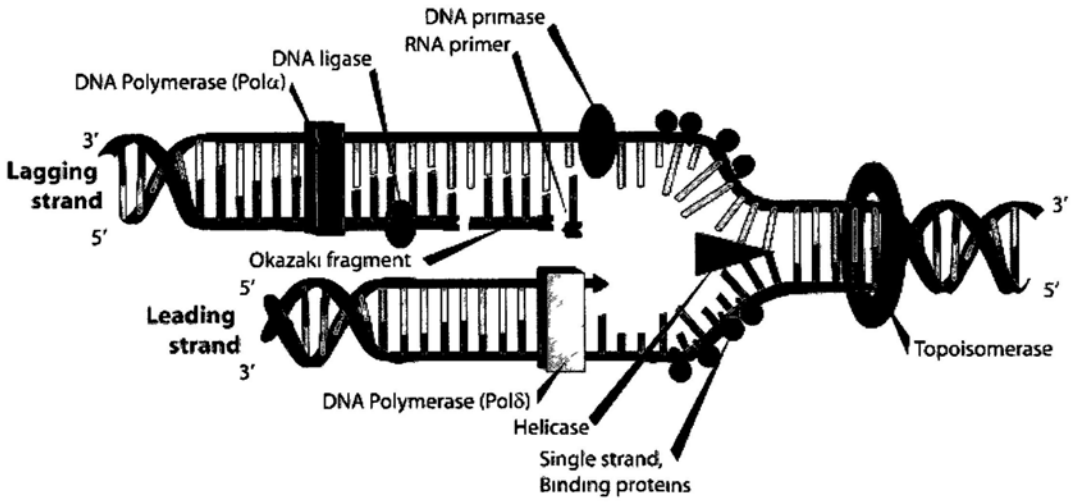

Figure 1.14. DNA replication at the "replicaton folk" on the Leading and Lagging Strands (photo adapted from Wikimedia Commons)

This process of replication repeated throughout the lagging strand until the last RNA primer attaches to the 3' end of the lagging strand. However, as there is no more polynucleotide chain ahead of it, DNA polymerase will not able to replicate the sequence. As a result of that, that region will not be replicated and the product chromosome will be shortened.

To overcome this problem, telomere which contains multiple repeated units (TTAGGG) acts a buffer region and located at the end of chromosome, a small fraction of it (TTAGGG repeat) will lose in each cell division so only repeat sequence will lose instead of genetic information. The telomere will also fold to a closed structure which call "t-loop" (Figure 1.14) to keep the DNA end from exposure. This telomere closed structure helps to maintain chromosomal stability,

and keep the end of polynucleotide chain from abnormality like cross fusion with other chromosome.

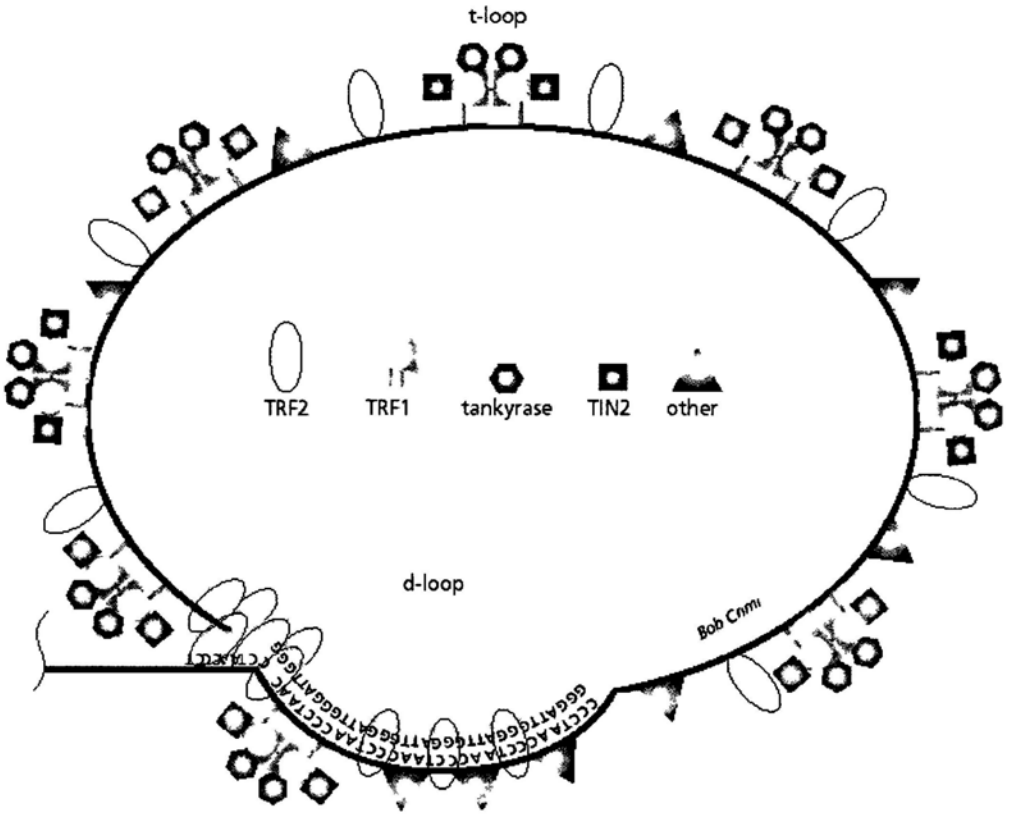

Figure 2. The t-loop structure of telomere at the end of chromosome (99).

With successive division, the telomere continues to be shortened until the length of it cannot hold the presumed t-loop structure and the end of the DNA are exposed. When it is happened, apoptosis will eventually occur.

c. Clinical Significance of Telomere

Due to telomere's physiological role in maintaining the chromosomal stability, any abnormality in the telomere length or structure may be detrimental. It can be

caused by many reasons like inherited abnormal telomere, dysfunction of telomerase or DNA-repair machinery of the cell (100-101). The most well-known of telomere-associated diseases is Dyskeratosis Congenita (DC). It is caused by mutation in telomerase complex which leads to decrease in the enzyme's function and resulted in abnormal shortening of the telomere. DC is known as premature aging and characterized by syndrome like skin pigmentation abnormalities, dystrophic nails and oral mucosa leukoplakia. Another disease which is also caused by mutation in telomerase is acquired aplastic anemia as a result of bone marrow failure. The telomere lengths of hematopoietic stem cells are short and therefore limit the proliferation ability of the stem cell and make the red blood cell production low.

Apart from telomerase mutation, unusual short telomere can be disease causing and it is one of the risk factors of cancerous conditions. When telomere is lost to a certain extent, it will be unable to hold the normal t-loop structure and provoke cell's DNA repair machinery. Under normal circumstances, p53 will be phosphorylated and lead to apoptosis (102). However, sometimes these cells may escape from the apoptosis and accumulate mutations which can lead to chromosomal instability. As a result, mutation will take place in telomerase gene and may lead to abnormal activation of telomerase. It had been reported that

almost 90% of tumors have telomerase dysfunction and telomere lengthening (100, 103).Short telomere is also a risk factor of heart disease, a number of studies indicate direct association between shortened telomere and myocardial infarction (100,101).

In the Nurses' Health Study, 62 participants were under association study to investigate whether there was any connection between telomere lengths in peripheral blood lymphocytes and dementia. The study showed that subjects with shorter telomere length were at higher risk in suffering dementia or mild cognitive impairment (MCI) and smaller hippocampus volume (104).

1.2 Early diagnosis of AD

AD causes great pressure not only to the patients, but also to their families and the health and welfare system. If diagnosis of the disease is made early, intervention measures may be enforced earlier to minimize complications. Although there is no effective medication to cure AD, intervention that may enhance quality of life and functioning are available.

The U.S. Food and Drug Administration (FDA) approve two types of drugs to for symptomatic management of cognitive symptoms of AD (105-106). Tacrine, donepezil, rivastigmine and galantamine are cholinesterase inhibitors that reduce

the rate of metabolism of acetylcholine, an important chemical messenger of synaptic signaling, thus enhances attention and episodic memory. Memantine is a low affinity irreversible N-methy-D asapartic acid (NMDA) receptor antagonist, which targets to regulate the glutamate activity. It has been approved for management of moderate to severe AD with documented efficacy in cognitive and social interactions. Different drugs have their own target disease stage and side-effects.

Generic	Brand	Approved For	Side Effects
donepezil	Aricept	All stages	Nausea, vomiting, loss of appetite and increased frequency of bowel movements.
galantamine	Razadyne	Mild to moderate	Nausea, vomiting, loss of appetite and increased frequency of bowel movements.
memantine	Namenda	Moderate to severe	Headache, constipation, confusion and dizziness.
rivastigmine	Exelon	Mild to moderate	Nausea, vomiting, loss of appetite and increased frequency of bowel movements.
tacrine	Cognex	Mild to moderate	Possible liver damage, nausea, and vomiting.

Table 1. A list of approved drugs for AD (data from the Alzheimer's Association)

1.3 Clinical and Biological Markers of Alzheimer's disease

1.3.1 Adjuvant Biological Biomarker

Cognitive function is a major diagnostic criterion for AD. It is also a reliable marker for early diagnosis. Apart from cognitive function, many biological markers reflecting neurodegeneration are under active research. At present, commonly reportedbiological markers include amyloid beta peptides, tau protein, hyperphosphorylated tau protein, and possibly ApoE 4 risk allele. Most protein biomarkers are extracted from cerebrospinal fluid (CSF), which represent an invasive approach to diagnosis (107-110).

Amyloid beta proteins

One of the hallmarks in pathological AD is the presence of amyloid plaque. Amount of soluble amyloid beta peptides (Aß42) in CSF is measured and many studies showed that normal people have the concentration of Aß42 exceeded 500pg/mL across all age groups. However, for people with AD, many studies identified that the level of soluble Aß42 dropped to about 50% of non-demented controls in the same age group (111-113). The decrease in soluble Aß42 level in AD patient is suggested to be caused by aggregation of amyloid proteins into insoluble plaques. For diagnostic classification, the CSF Aß42 test was reported to have a sensitivity and specificity of around 85% and 55% (112) to indentify AD from other disorders not associated with pathologic conditions of the brain and

non-Alzheimer types of dementia (NAD).

Tau proteins

Apart from amyloid proteins, tau protein deposition is another hallmark of AD. Under common circumstances, tau proteins are appeared as intracellular tangles (NFT), and are essential for axonal transport. In AD and other neurodegenerative disorders, tau proteins are present in CSF as a result of release from neuronal death (113-114). The assays of total tau proteins for AD diagnosis include both phosphorylated and usual tau.

The tau protein can be used as a biomarker to discriminate AD from other dementia and achieved a better specificity of 65% than standalone amyloid beta test at 85% sensitivity (112) when trying to discriminate AD from NAD.

Hyperphosphorylated tau proteins

Similar to total tau proteins test, hyperphosphorylated tau proteins are also found in CSF. The advantages over total tau proteins test is that it can detect AD from some other dementia like semantic dementia and frontotemporal dementia with better sensitivity and specificity. The sensitivity and specificity of total tau test are 66% and 88.9% respectively, while that of phosphorylated tau test are

71.7% and 92.4% (112).

ß-secretase and ApoE 4 risk allele

ß-secretase is one of the enzymes that cleave the amyloid precursor proteins (APP) and initiates the production of A*ß (115).* However its activity will drop in AD brain and make it as a potential biomarker due to significant variation in AD subjects when compared with normal control (116). Apart from ß-secretase, ApoE4 allele is well-known as a risk factor for AD. However, the sensitivity and specificity of ApoE4 allele test of AD patients are low, as it only adds a 5-10% confidence to AD diagnosis (117). The test combined with the detection of ß-secretase and ApoE4 allele can contribute additional certainty of other diagnostic tools with classification accuracy of 78%. The sensitivity was 80% and the specificity was 77% (118).

Biomarkers mentioned above require extraction of CSF which may be unacceptable to persons with MCI, the at-risk but not yet clinical dementia. It will be more user-friendly if the biomarkers, with high sensitivity and specificity, would be available through peripheral blood. Thus, there is a need for continue search for biomarkers in peripheral blood.

1.3.2 Neuroimaging markers

Magnetic resonance imaging (MRI)

High resolution MRI is used to provide structural information of the brain (119-120). MRI uses a strong magnetic field to align the nuclear magnetization of hydrogen atom to produce a shift in magnetic field which is detected by the scanner. MRI shows cell loss in brain regions by providing image of shrunken structure in the brain. For diagnosis of AD, decreased hippocampal volume indicated by MRI in people with MCI is a predictive factor for disease progression to AD (accuracy up to 80%).

Positron Emission Tomography (PET)

Similar to MRI, PET is also an imaging technique and able to produces a three-dimensional image (120-122). Unlike magnetic field detected by MRI system, PET system use gamma ray emitted by a positron-emitting tracer, which can be biological active molecule, to reconstruct structural images. PET can use different types of tracers and the choice is depended on the metabolic activity of interest. ForAD, (18FDG) 18-fluorodeoxyglucose and PIB ([N-methyl-11C]2-[4'-(methylamino)-phenyl]6- hydroxybenzothiazole) are commonly used as the tracer of PET. 18FDG-PET is used to investigate cortical

metabolism. MCI patients show a reduced cortical uptake in temporal region and parietal association cortex. The cortical uptake is further lowered when MCI progress to AD and therefore 18FDG-PET can be used as an early AD diagnostic tool (123).

PIB is also used as tracer but instead of being a metabolic substance, it passes through blood-brain barrier and binds to amyloid in the brain. PIB may detect the location and amount of amyloid plaques in the brain, and the PIB-PET has been demonstrated to show good sensitivity in detecting AD pathological changes in subjects with MCI. Its predictive ability for disease progression remains further investigation.

CHAPTER 2. Aims and Hypothesis of the Study

2.1 Aims of the Project

Considering the limits of identifying persons suffering from early pathological AD with only clinical and cognitive assessment, it is important to explore adjuvant markers that would help to offer additional information for early diagnosis. Extensive researches have been conducted in this area. Neuroimaging and neurochemical markers were reported to offer reasonable differentiating power for early AD (120-122). However, they are relatively expensive, may be invasive and demand sophisticated assessment techniques thus limiting routine use in clinical practice. The aim of my research project is, therefore, to identify potential biomarkers that are non-invasive but yet valid and reliable.

Currently most neurobiological markers, such as phosphrylated Tau (116-117), are extracted from cerebrospinal fluid (CSF). The CSF extraction required well-trained personnel to carry out and a local anesthetic medicine is administrated to the patient to minimize discomfort. As CSF extraction is a relatively invasive procedure, it is not likely to be used as a routine measure in this locality.

Peripheral blood sampling offers many types of diagnostic targets, i.e. metabolic products, proteins, mRNAs and genomics DNAs. Different clinical studies aimed to

build different profiles of biomarkers that would be potential useful for early diagnosis of AD. A recent study found out 18 signaling proteins, e.g. IL-3, IL-11, EGF etc., in blood plasma that combination analysis of their expression levels by ELISA produced high accuracy to identify MCI patients who may progress to AD (124). Another study discovered showed that expression level variations in 4 out of 33 genes quantified by RT-PCR were correlated with MMSE scores (125). The 4 genes were sorting nexin II (SNX2), histone cluster 1 H3e (HIST1H3E), cannabinoid receptor 2 (CNR2) and glutamate receptor, ionotropic, Kainate 4 (GRIK) genes.

Apart from proteins and mRNAs, single nucleotide polymorphisms (SNP) are also found to be associated with AD. The most well replicated is the polymorphisms of ApoE4 gene, i.e. rs6265, rs11030104, and rs2049045 (126). Many different gene polymorphisms have also been reported to have associations with AD, although the reliability is much lower than Apo E4. These studies unveiled the feasibility of employing genetic markers obtained from peripheral blood for diagnostic purposes.

As reported in the Introduction, my project aimed is to explore if differential factors related to cell cycle regulations and cellular viability could serve as potential peripheral markers for early diagnosis of AD. In this thesis, I would focus on the investigation of associations between (1) cell cycle gene polymorphisms and (2) telomere lengths, global cognitive function and the risks of developing AD.

2.2 Hypotheses

With reference to the literature review about potential relevance of cell cycle checkpoint genes and telomere in the pathogenesis of AD, the present project hoped to address the following study objectives:

1. Association between single nucleotide polymorphism (SNP) of cell cycle checkpoint genes, and diagnosis of AD
2. Association between telomere lengths with aging and global cognitive function.

The following null hypotheses were set for this project.

1. There would be no association between cell cycle check point gene polymorphisms and diagnosis of AD.
2. There would be no association between telomere lengths and age.
3. There would be no association between telomere length and global cognitive function.
4. There would be no association between telomere lengths and diagnosis of AD

CHAPTER 3. Methodology

The thesis presented 3 related studies addressing the above hypothesis: 1) Association study of Cell Cycle checkpoint Genes SNPs and AD; 2) Association study of telomere lengths and global cognitive function in a clinical sample of AD .; 3) Association study of Telomere lengths and global cognitive function in community dwelling healthy men in healthy aging study.

3.1 Association study of Cell Cycle checkpoint Genes SNPs and AD

3.1.1 Samples recruitment

AD patients were recruited at the psychogeriatric clinic and outreach facilities of the New Territories East Cluster (NTEC) hospitals in Hong Kong. Those satisfied inclusion criteria were recruited and with consent obtained from subjects or first degree relatives. The selection criteria included matching the NINCDS-ADRDA criteria for probable or possible AD, absent of serious head injury or predisposing medical disorders in their medical history, had the ability to complete the assessment and consent for genetic analysis of their blood samples.

Cognitively intact control subjects were recruited from local social centers and were assessed by a qualified psychiatrist for their cognitive status. In this study,

there were 255 AD patients and 446 non-demented subjects. The average age in years of AD group was 81 (S.D. =7.3; Range=51-97) and that of normal group was 71 (S.D. =7; Range=57-96). For gender distribution, there were 65.9% female in the control group, with 85.3% were female in the AD group.

3.1.2 Blood Sample Collection

Blood sample was collected by venepuncture and stored in EDTA tubes. A volume of 9 ml was taken. Blood samples were transported to laboratory for genomic DNA extraction as soon as possible.

3.1.3 Genomic DNA Extraction

Commercial genomic DNA kit (Biogene, Korea) was used to extract genetic materials from blood samples. Procedures were carried out according to manufacturer's protocol.

The blood samples were centrifuged at 1500 X g for 10 minutes at 18℃ and plasma was removed. The white blood cells in buffy coat were lysed by protease K and separated from proteins by centrifuging at 13000rpm. After that the DNA was bound to resin and washed with buffer provided in the kit. Finally the DNA was eluted in water.

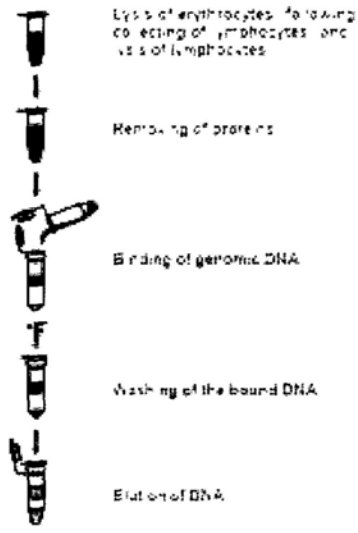

Figure 3.1 A brief workflow of DNA extraction from blood by a commercial kit.

The concentration of DNA extracted was measured by NanoDrop Spectrophotometer (Thermo Fisher Scientific, USA) and most of DNA samples were with a concentration more than 100ng/ml. The quality of extracted DNA was checked with reference to the ratios of 260nm/230nm and 260nm/280nm which indicated polysaccharide and protein contaminations respectively. Both ratios of 260nm:230nm and 260nm/280nm that were greater than 1.7 represented a good DNA quality with low level of contaminations. All DNA samples were stored in 96-well plates with 88 samples on each plate as column 12 left empty for control if necessary.

3.1.4 Target Cell Cycle Checkpoint Genes

10 genes that play important regulatory role in cell cycle were studied in this

project. These included: RB1, CDKN1A, CDK5R1, CDK2AP1, CDKN2A, CDKN2C, MDM2, P53, GSK3B, TPND1 and CDKN1B.

3.1.4.1 Retinoblastoma 1 gene (RB1)

RB1 gene is located on chromosome 13q14.1-14.2. There are 13 SNPs including 5k base pairs areas upstream and downstream of the gene, i.e. rs1573601, rs520342, rs4151450, rs399413, rs9526475, rs9568036, rs198607, rs198604, rs1894255, rs9535032, rs1951774, rs198584 and rs990814.

The RB1 protein plays an important role in cell cycle regulation and is responsible to control progression from G1 phase to S phase. pRB1 functions to inhibit cell replication when it is hypophosphorylated by binding to transcription factor E2F which can lead to downstream mitotic pathway activation. The pRB1-E2F complex also induces histone deacetylase (HDAC) to chromatin and helps to reduce DNA synthesis to prevent unregulated start of S-phase. The E2F will be released when G1 phase is finished and pRB1 is phospohrylated.

The phosphorylation of pRB1 was shown to be related to AD. The phosphorylated pRB1 redistributed from nucleus to cytoplasm of susceptible neurons and co-localized with amyloid plaque and neurofibrillary tangles (127). Apart from the linkage of phosphorylated pRB1 to late-onset Alzheimer's disease, the SNP

rs1573601 of the RB1 gene was found to be associated with early-onset AD (90).

3.1.4.2 Cyclin-dependent kinase inhibitor 1A gene (CDKN1A)

CDKN1A is also known as p21/Waf1. The gene is located on chromosome 6p21.2. There are 6 tag SNPs including 5k base pairs areas upstream and downstream of the gene, i.e. rs3176323, rs10947623, rs1801270, rs12207548, rs3176343 and rs2395655.

CDKN1A (or p21) is an important G1 cell cycle checkpoint regulator and works as a cyclin-dependent kinase inhibitor (CKI) (128). The main function of p21 is to bind and inhibit cyclin-CDK2 and cyclin-CDK4 activities (129). This prevents cyclin-CDK complexes to phosphorylate downstream proteins and arrest the cell cycle in G1 phase. It can interact with PCNA (proliferating cell nuclear antigen) and regulate DNA replication at the start of S-phase (130). p21 may lead to cell apoptosis in response to stress when it is cleaved by caspases and induce activation of CDK2. p21 was included as its role in controlling downstream cyclin-dependent kinase may probably contribute to tau phosphorylation (131).

3.1.4.3 Cyclin-dependent kinase 5 activator 1 gene (CDK5R1)

The gene is located at the position chr17:27.84-27.84Mb. There are 9 SNPs

including 5k base pairs areas upstream and downstream of the gene, i.e. rs2286246, rs2285428, rs4523973, rs731880, rs756785, rs756787, rs8069868, rs9333303 and rs9889771.

The CDK5R1 encodes protein p35 and lead to activation of cyclin-dependent kinase 5 (CDK5) in neuronal cell (132). The p35 protein is cleaved by calpain to generate a p25 form. Studies showed that p25 lead to CDK5 activity deregulation by extending its kinase activity. It is found that p25 proteins are accumulated inside neurons of patients suffering from AD, and the degree of p25 accumulation is positively correlated with the activity of CDK5, which is found to contribute to tau protein hyperphospohrylation (132).

3.1.4.4 Cyclin-dependent kinase 2-associated protein 1 gene (CDK2AP1)

The gene of CDK2AP1 is positioned at chromosome 12 (location: chr12:122.31-122.32Mb). There is only 1 tag SNP including 5k base pairs areas upstream and downstream of the gene, i.e. rs3759114. The protein CDK2AP1 specifically binds to CDK2 (cyclin dependent kinase 2) and inhibits its activity. CDK2AP1 is also found to be involved in maintaining DNA methylation and studies show that AD susceptible neuron has abnormal low expression of factors related to DNA methylation and methylation maintenance.(134). In AD neurons,

immunostaining experiment suggested that DNA methylation level was dramatically decreased and gene expressions were abnormal (134). Apart from that, CDK2AP1 interacts with DNA polymerase and plays a regulatory role in S phase DNA replication (135).

3.1.4.5 Cyclin-dependent kinase inhibitor 2A gene (CDKN2A)

The location of the gene is in chromosome 9 and position 21.96-21.98Mb. The protein product CDKN2A is also called p16. There are 5 tag SNP including 5k base pairs areas upstream and downstream of the gene, i.e. rs2811712, rs3731257, rs2811710, rs3731217 and rs3218020. p16 is a G1 phase checkpoint protein. It interacts and forms a complex with MDM2 in order to prevent degradation of p53 by MDM2 (136). Also it is a CDK inhibitor and regulates the activity of CDK4 (137). In non-demented brain neuron, p16 is not expressed. However, when AD neuronal cells are examined, it was found out that p16 is presented in high level (138). The abnormal expression of p16 may disrupt normal cell cycle progression and contribute to cell death.

3.1.4.6 Murine double minute 2 gene (MDM2)

The MDM2 gene is located at the position chr12:67.49-67.52Mb. It encodes 3

proteins, i.e. MDM1, MDM2 and MDM3. There are 3 tag SNPs including 5k base pairs areas upstream and downstream of the gene, i.e. rs2870820, rs3730581 and rs1695147.

MDM2 plays several functions in cell cycle and is a very important regulator. It regulates the activity of p53 by a controlled degradation manner (139). Apart from that, MDM2 also promotes degradation of pRB. As p53 and pRB are important cell cycle regulators in G1 checkpoint and both contribute significantly to correct DNA replication during mitosis, abnormal MDM2 expression can lead to mutation accumulation or re-entry of cell cycle of neurones (131).

3.1.4.7 Tumor protein p53 gene (p53)

The protein p53 is encoded by the gene TP53 located at chromosome 17:7.51-7.53Mb. There are 3 tag SNPs including 5k base pairs areas upstream and downstream of the gene, i.e. rs17882227, rs4559962 and rs12951053.

The protein p53 is a G1/S checkpoint regulator. In normal cells, it is suppressed by MDM2 and the cell progresses to S phase. However when there are DNA damage, cell cycle abnormalities or external stress, p53 will be released by MDM2. Free p53 induces the transcription of the protein p21. The p21 protein will bind to CDK2 and then stop the mitosis at G1 phase and prevent it to start S phase. The cell which is

hold in G1 phase starts the DNA repair process and make sure all abnormalities are fixed before cell cycle is progressed to S phase. Also in some case when the DNA repair mechanism fails, the cell will undergo apoptosis (139).

Studies show that the expression level of p53 in neurons of patients with AD is much higher than that of normal elderly (140). Also p53 is found out to have an indirect effect to tau protein hyperphosphorylation (141).

3.1.4.8 Glycogen synthase kinase 3 beta gene (GSK3B)

The GSK3B gene is located at chr3:121.03-121.3Mb. There are 4 tag SNPs including 5k base pairs areas upstream and downstream of the gene, i.e. rs10934503, rs16830594, rs2199503 and rs13321783. The GSK3B protein interacts with p53 and increases the kinase activity of GSK3B. In normal cells, the p53 protein exists in a low level and GSK3B will phosphorylate MDM2. Phosphorylated MDM2 leads to degradation of p53 when binding to it. However under cellular stress, MDM2 will dissociate from MDM2-p53 complex and free the p53 protein. The high level of p53 will bind to GSK3B and hence increase the activity of kinase. GSK3B phosphorylates tau proteins and increases production of amyloid (142). Amyloid precursor protein and Aß can bind to the promoter of p53 and activate transcription of it. The combination effect of high level of p53 and GSK3B is a positive feedback loop and

may contribute significantly to the pathology of AD (143-144).

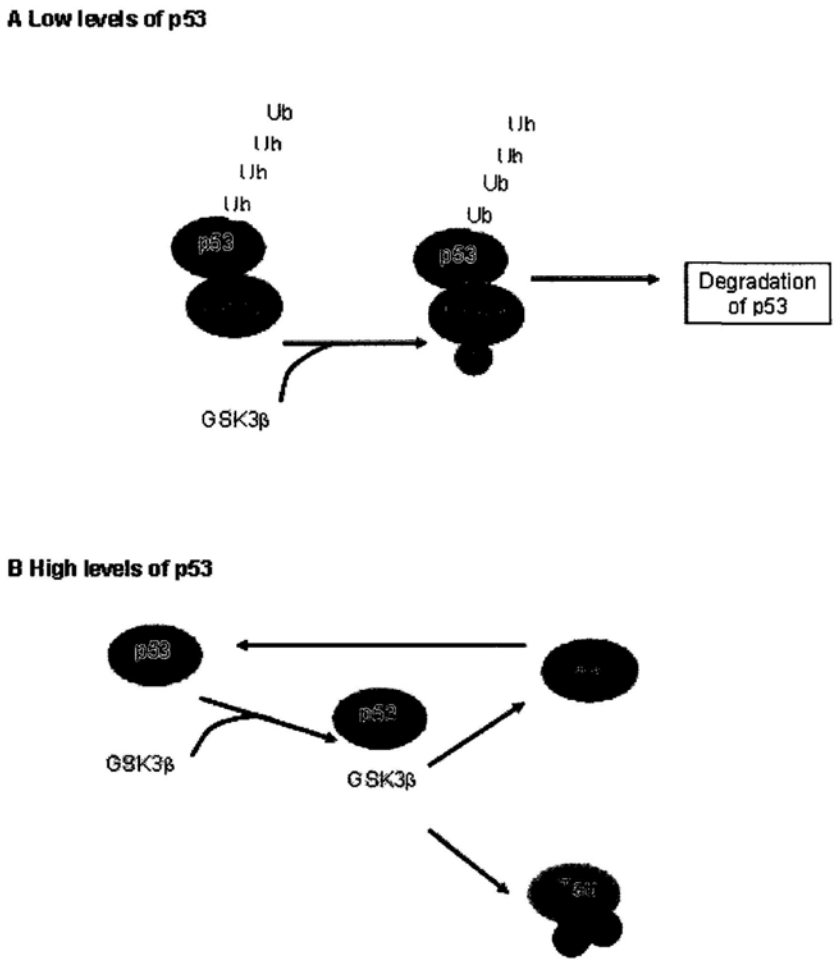

Figure 3.2(A) Breakdown of p53 in normal cells to maintain a relative low level of p53. (B) High level of p53 in stressed cell cause GSK3B to phosphorylate tau and activate Aß production (102).

3.1.4.9 Cyclin-dependent kinase inhibitor 1B gene (CDKN1B)

The gene is located at the position chr12:12.76-12.77Mb. There are 4 tag SNPs including 5k base pairs areas upstream and downstream of the gene, i.e. rs34330, rs34322, rs12229100 and rs3759216.The protein CDKN1B is a cyclin dependent

kinase inhibitor and inhibits the activation of cyclin E-CDK2 or cyclin D-CDK4 complex. Therefore it belongs to one of the cell cycle G1 checkpoint regulators. The CDKN1B causes the cell to hold in G1 phase by preventing the CDK4 to adding phosphate to RB1 protein (145).

3.1.4.10 Transcription factor Dp-1 gene (TPDP1)

The protein is encoded by the TFDP1 gene located in the chromosome 13. There are 4 tag SNPs including 5k base pairs areas upstream and downstream of the gene, i.e. rs4150715, rs4150735, rs9577581 and rs12867405. The TPDP1 plays its regulatory role as a subunit of E2F-TPDP1 complex which binds to promoters and controls the expression of various cell cycle related proteins (146-147).

3.1.5 Single Nucleotide Polymorphism (SNP) Analysis

3.1.5.1 Primer Design

A web-based software was used to design mismatch primer for each SNP. The software was dCAPS Finder 2.0 developed by Neff in 2002 (148).

dCaps Finder 2.0 Output

Number of Mismatches in the primer: 1

Wild Type Forward: AGAAAGATTTCTGAAGTTAGGTAGGAATGGCTGTTGAGTAGGCCAGTGCTTG
Mutant Forward: AGAAAGATTTCTGAAGTTAGGTAGGACTGGCTGTTGAGTAGGCCAGTGCTTG

Wild Type Reverse: CAAGCACTGGCCTACTCAACAGCCATTCCTACCTAACTTCAGAAATCTTTCT
Mutant Reverse: CAAGCACTGGCCTACTCAACAGCCAGTCCTACCTAACTTCAGAAATCTTTCT

These matches were found for:

Cutting wild type forward sequence:

ENZYME	RECOGNITION SEQUENCE	PRIMER SEQUENCE
BsrDI :	GCAATG	AGAAAGATTTCTGAAGTTAGGTAGCA
FokI :	GGATG	AGAAAGATTTCTGAAGTTAGGTAGGG
NlaIII :	CATG	AGAAAGATTTCTGAAGTTAGGTAGGC
HinfIII :	CGAAT	AGAAAGATTTCTGAAGTTAGGTACGA

Cutting wild type reverse sequence:

ENZYME	RECOGNITION SEQUENCE	PRIMER SEQUENCE
TfiI :	GAWTC	CAAGCACTGGCCTACTCAACAGCGA
TspEI :	AATT	CAAGCACTGGCCTACTCAACAGCAA

Cutting mutant forward sequence:

ENZYME	RECOGNITION SEQUENCE	PRIMER SEQUENCE
Cac8I :	GCNNGC	AGAAAGATTTCTGAAGTTAGGTAGGG
CviJI :	RGCY	AGAAAGATTTCTGAAGTTAGGTAGGC
EcoRII :	CCWGG	AGAAAGATTTCTGAAGTTAGGTAGGC
FinI :	GGGAC	AGAAAGATTTCTGAAGTTAGGTGGGA
HaeI :	WGGCCW	AGAAAGATTTCTGAAGTTAGGTAGGC
HaeIII :	GGCC	AGAAAGATTTCTGAAGTTAGGTAGGC
RsaI :	GTAC	AGAAAGATTTCTGAAGTTAGGTAGTA
ScaI :	AGTACT	AGAAAGATTTCTGAAGTTAGGTAGTA
SerFI :	CCNGG	AGAAAGATTTCTGAAGTTAGGTAGGC
StuI :	AGGCCT	AGAAAGATTTCTGAAGTTAGGTAGGC
TatI :	WGTACW	AGAAAGATTTCTGAAGTTAGGTAGTA
TspRI :	CASTGNN	AGAAAGATTTCTGAAGTTAGGTAGCA

Figure 3.3 A sample output of dCAPS Finder 2.0 with suggested restriction enzymes on the left side and primers on the right side.

After the mismatch primer was decided, the remaining reverse primer was designed using another online tool, i.e. Primer3, developed by Steve Rozen in 2000.

Physical properties of each primer in the primer pair were determined by an

online oligonucleotide properties calculator- Oligo Calc (149). The melting temperature of each primer pair was controlled to fall into a range between 55℃ to 62℃ and sequences were checked to prevent self-complementarities.

All primers were ordered from a local company TechDragon Ltd. The primer came in a stock concentration of 100mM and was dilute 10 fold to 10mM using double distilled water.

3.1.5.2 Amplification of SNP Containing Sequence

Polymerase chain reaction (PCR) was carried out with each pair of SNP-specific primer. Each reaction mix contained the following components:

(1) 2µl of 10X Taq buffer with $(NH_4)_2SO_4$,

(2) 1.6µl of 25mM $MgCl_2$,

(3) 0.5µl of 10mM dNTP mix,

(4) 0.5µl of 10mM forward primer

(5) 0.5µl of 10mM reverse primer

(6) 5µl of DNA template (stock concentration: 10ng/µl)

(7) 0.2µl Taq polymerase (Fermentas, 5units/µl)

(8) 9.7µl of ddH_2O

The total reaction volume was 20µl.

Afterwards, the reaction mixtures were placed inside a Bio-Rad iCycler 96-well PCR machine.

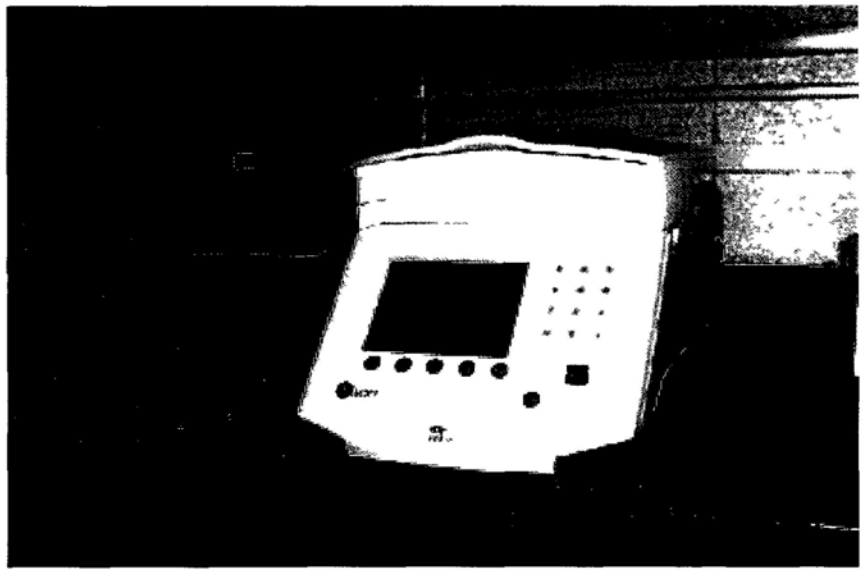

Figure 3.4 Bio-Rad iCycler PCR System.

The PCR reaction was set to preheat the reaction mixtures to 96℃ for 15 minutes and then followed by 35 cycles of (1) 96℃ for 20 seconds, (2) 55℃ for 30 seconds and (3) 72℃ for 40 seconds. Lastly the reactions were kept at 72℃ for 15 minutes and cooled down to 4℃.

3.1.5.3 Digestion with Restriction Enzyme

After finishing the PCR, 3μl of products were loaded in a 4% agarose gel (Biowest, Spain) to check the quality. A strong band with the size around 60bp and absent of other unrelated bands represented a good quality and the PCR products could be subjected to enzyme cutting.

Each mismatch primer pair had a predesigned restriction enzyme cutting site which was allele-specific. 10µl of PCR products and reaction buffer (NEB, UK) were added to the reaction mixture with 3units of restriction enzyme (NEB, UK). Water was filled until reaction volume reached 20µl. The reaction mixture was placed at the optimum working temperature of the enzyme for overnight.

The digested reaction product was loaded on a 4% agarose gel and the genotype of the SNP was determined from the size pattern of digested fragments. The digested fragments pattern on the gel was visible by staining of ethidium bromide. There were 3 types of patterns which represented the genotype HH, HL or LL that "H" was the indigestible allele and "L" was the one could be cut.

To make sure all samples were cleaved correctly with accordance to their genotypes, eight Chinese samples were chosen from the 45 HapMap Chinese sample panel which composed all 3 haplotypes were added on each experimental plate. The haplotype data of all 45 Chinese samples can be obtained from HapMap database. The results were accepted only if those 8 Chinese control samples were correctly cut.

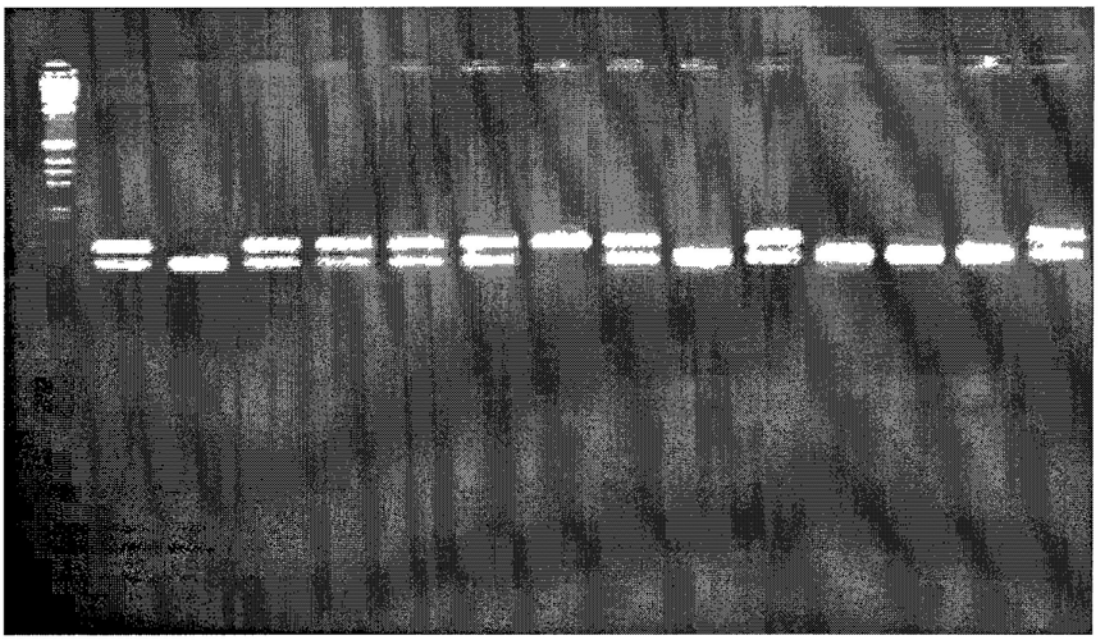

Figure 3.5 An agarose gel photo of restriction enzyme digested products. The leftmost lane was DNA ladder, the lane next to it was "HL" and then followed by the "LL" genotype. The eighth lane from the left was the genotype "HH".

3.2 Telomere Length Measurement in a clinical sample of AD

3.2.1 Principle of the measurement

The principle of telomere measurement was based on the corrected Ct ratio of telomere and control gene obtained from quantitative real-time PCR, i.e. T/S ratio. In the experiment, 36B4 was chosen to be the control gene. The T/S ratio was made reference to the ratios of standard curve established from subjects with measured telomere length.

3.2.2 Subjects Recruitment

Cognitively normal elders and subjects with AD were recruited from the psychogeriatric clinics and elderly community centers for the cell cycle checkpoint gene polymorphism study. Details can be referred to section 3.1.1.

3.2.3 Determination of Telomere Length of Control Subjects

There were four control subjects included, three of them were chosen from local Chinese elderly (HK1007, HK1880 and HK1908) and the remaining was come from a young woman (Control-R). The approach used to quantify those controls was terminal restriction fragment (TRF) analysis and a commercial kit TeloTAGGG Telomere Length Assay of Roche was used.

Around 0.5μg of genomic DNA from the controls were subjected to cutting by Hinf I and Rsa I restriction enzymes. The reason of using Hinf I and Rsa I in the TRF analysis was that their respective DNA recognition sites GANTC and GTAC were very common in the genome. As a result of that of the genomic DNA were cleaved into many pieces of polynucleotide chains. However telomeres at the end of chromosome were exempted from cleavage because of its repetitive sequence TTAGGG contained no restriction site for the enzyme. At the end of enzymatic digestion, telomere would retain its original length, which existed as TRF, and other genomic DNA was cleaved.

genomic DNA was cleaved.

The HaeIII cleaved reaction products were loaded in an agarose gel and the telomere fragments were separated by gel electrophoresis. The fragments in the agarose gel were transferred and bound to a nylon membrane by Southern blotting. The fragments bound on the membrane were then hybridized with a digoxigenin (DIG)-labeled oligonucleotide probe. The probe had the sequence TTAGGG which was specific to repetitive telomere sequence.

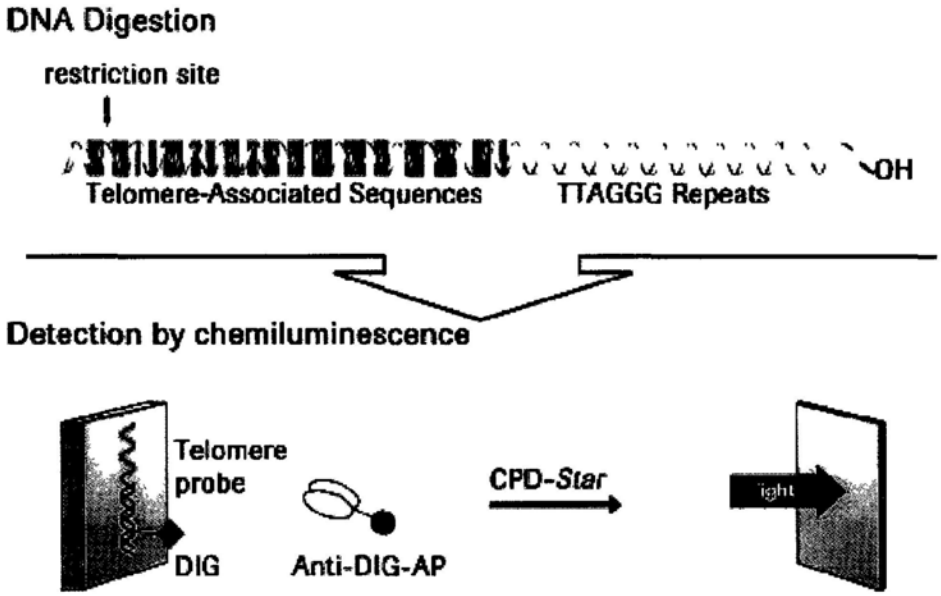

Figure 3.6 Working principle of the Roche TeloTAGGG Telomere Length Assay.

The membrane with bound labeled fragments was incubated with DIG-specific antibodies covalently coupled to alkaline phosphatases. As the TRFs were labeled with DIG, the antibodies bound to the fragments and the alkaline phosphatases coupled to the antibodies metabolized the chemiluminescent substrate, CDP-Star.

PhosphoImager (Molecular Dynamics) and the size of telomere of the control could be determined by comparing to the molecular-weight standard.

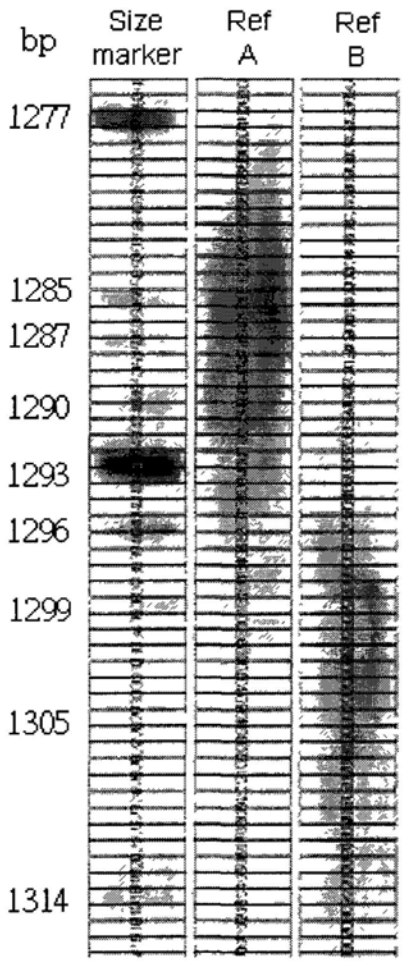

Figure 3.7 The smears in the lanes Ref A and Ref B represented the TRF and the sizes of the smears were compared with marker.

3.2.4 Quantitative Real-time PCR (qRT-PCR)

The real-time PCR was used to measure the length of telomere of samples. The PCR reaction was done on the Roche LightCycler 480 and AmpliTaq Gold DNA polymerase from Applied Biosciences.

polymerase from Applied Biosciences.

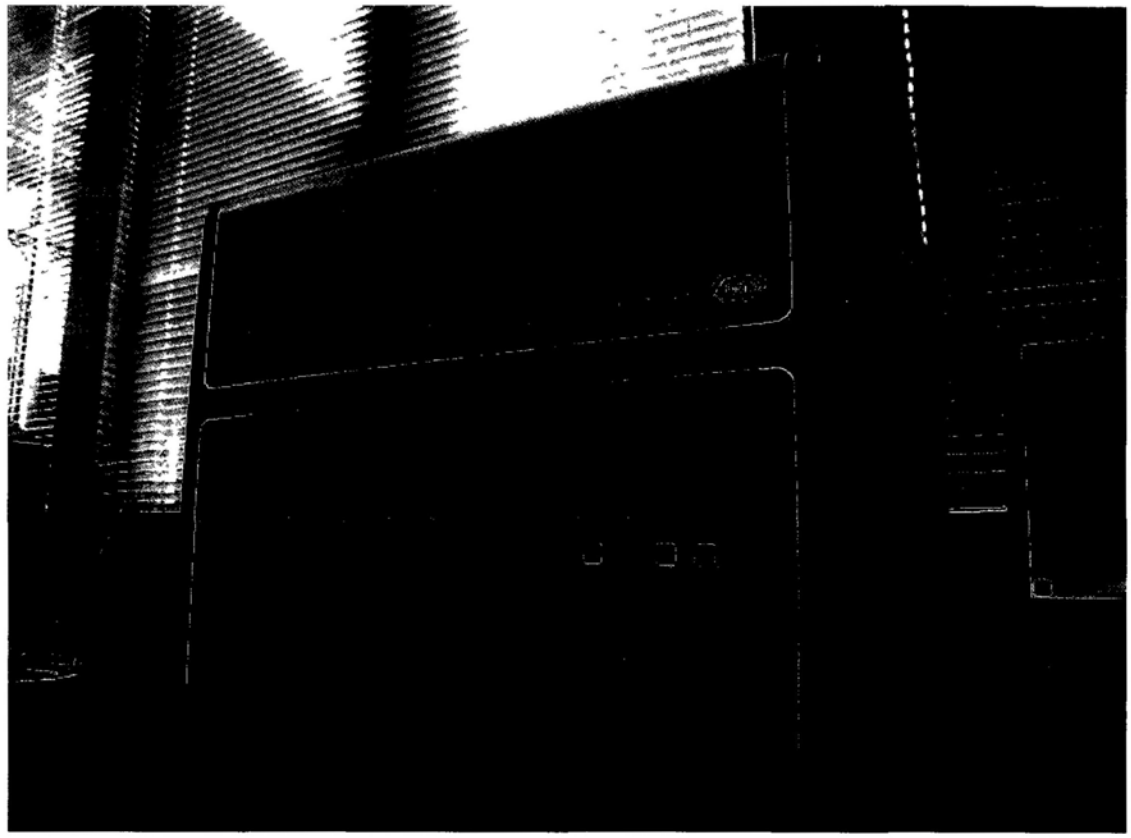

Figure3.8 Roche LightCycler 480 qRT-PCR machine

The reactions were performed in 386-well plate. DNA of the controls (HK1007, HK1880 and HK1908) and four serial dilutions of 10ng/µl, 5ng/µl, 2.5ng/µl and 1.25ng/µl of Control-R were added on the plate as quality controls and reference samples.

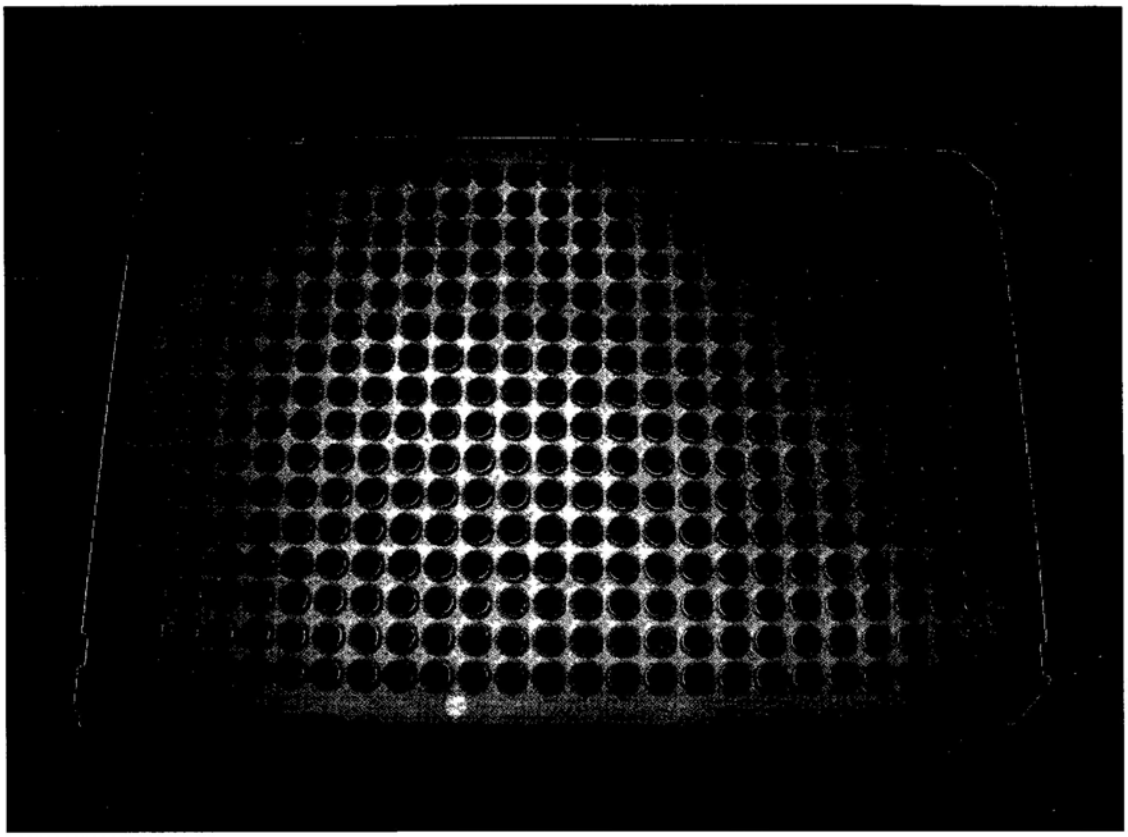

Figure 3.9 A 384-well PCR pate manufactured by Roche and used in the qRT-PCR

In order to obtain the T/S ration, all samples should be tested with a single copy internal control gene apart from the telomere and the gene 36B4 was chosen as the control gene. As a result of that, all samples were required to perform two sets of reaction, i.e. telomere measurement assay and control gene assay. The reaction mixtures of the telomere measurement assay and control gene assay contained similar components except using different primers (sequences listed below).

Primer sequences for the telomere measurement assay:

(1) tel1b, 5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3';

(2) tel2b, 5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'.

Primers sequences for the control gene assay:

(1) 36B4u, 5'-CAGCAAGTGGGAAGGTGTAATCC-3';

(2) 36B4d, 5'-CCCATTCTATCATCAA CGGGTACAA-3'

The primer set for telomere measurement assay (tel1b & tel2b) was designed to bind to the telomere repetitive sequence TTAGGG and its antisense sequence CCCTAA while the primer set for control assay (36B4u & 36B4d) was used to bind and amplify the gene of acidic ribosomal phosphoprotein P0 (36B4) which was a single copy gene.

Each reaction mixture contained the following components.

(1) 50ng of DNA sample

(2) 2µl of 10X Reaction Buffer

(3) 1.6µl of 25mM $MgCl_2$

(4) 0.5µl of dNTP mix

(5) 0.5µl of forward primer

(6) 0.5µl of reverse primer

(7) 5 units of AmpliTaq Gold DNA polymerase

(8) H_2O added to final volume of 20µl

The amplification steps of the qRT-PCR were set as follow:

(1) 95℃ for 10 minutes

(2) 95℃ for 15 seconds

(3) 60℃ for 60 seconds

(4) Repeat Step (2) and (3) for 30 cycles

(5) 72℃ for 15 minutes

(6) Hold at 4℃

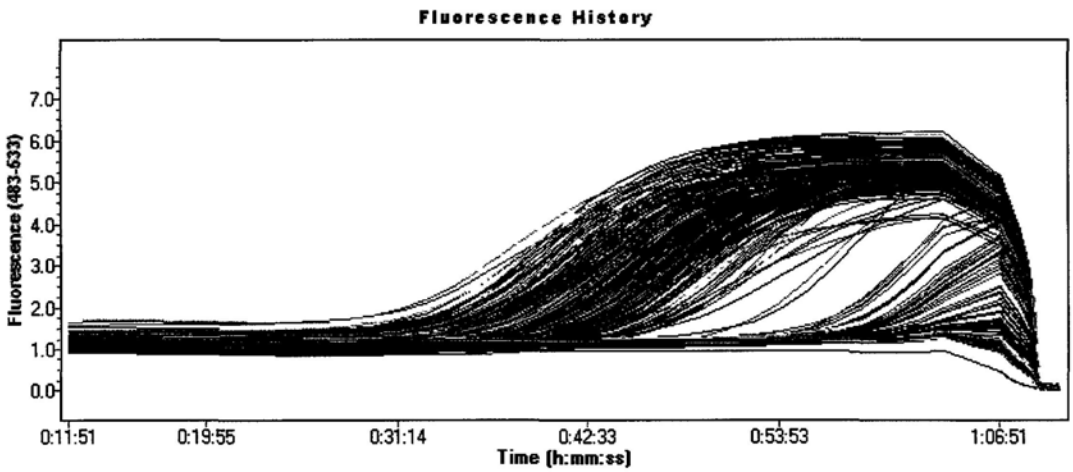

Figure 3.10 The amplification curve in qRT-PCR

The quantity of telomere amplified product of each sample was represented by the respective Ct value. The Ct values of telomere measurement assay and control assay of each sample could be used to calculate the sample's T/S ratio. However, the quality of PCR products were checked before those obtained data could be used as

we needed to make sure the PCR amplified the targeted genes with no other contamination. Melting curve of the amplified product was examined for every sample and products were put under analysis by gel electrophoresis.

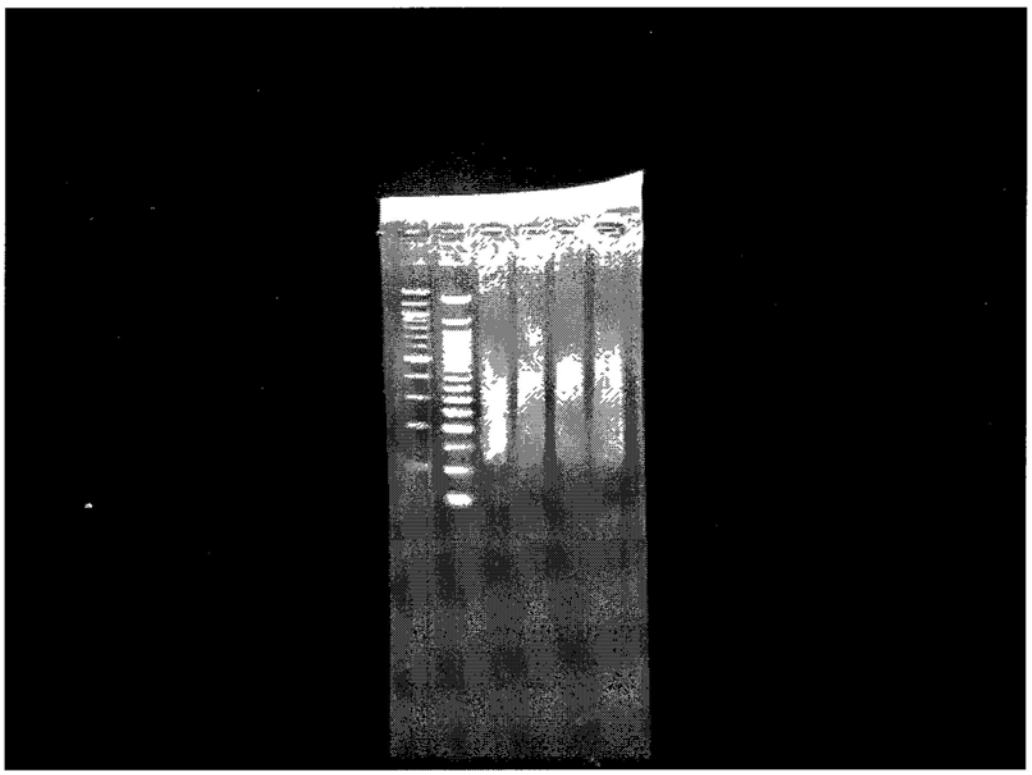

Figure 3.11 Photo of gel electrophoresis of telomere amplified products by qRT-PCR. The smears represented different amplified telomere repeats in various lengths.

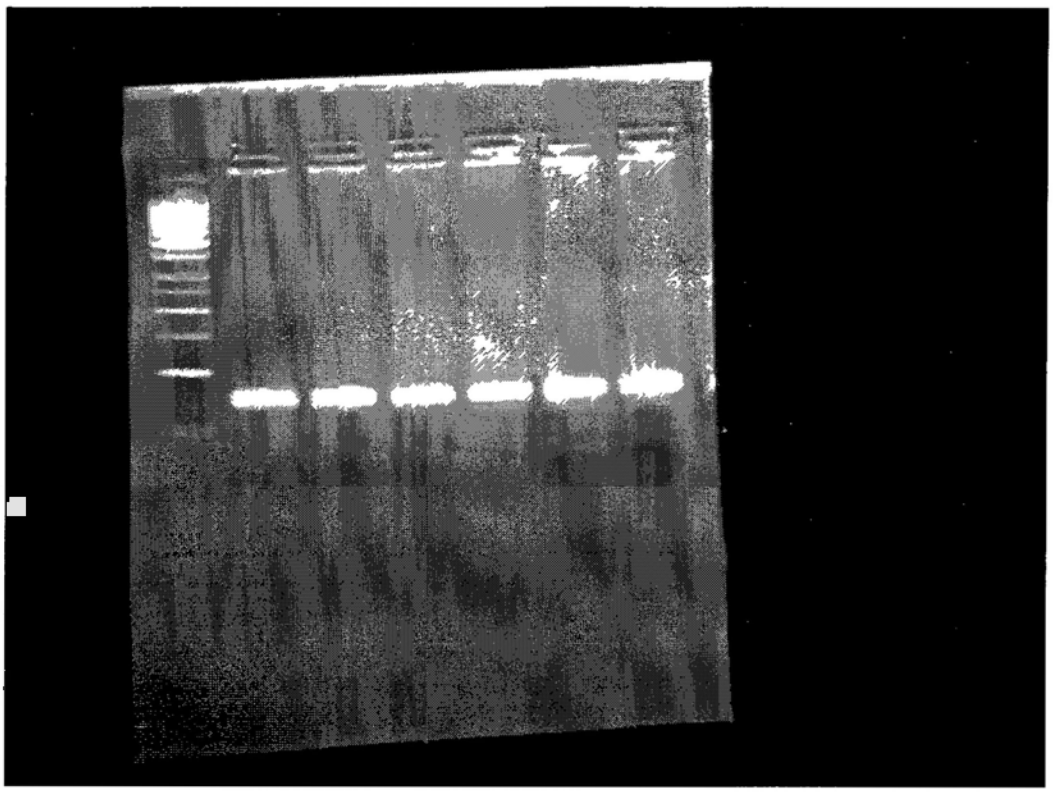

Figure 3.12 Photo of gel electrophoresis of qRT-PCT amplified products of the 36B4 single copy control gene. The leftmost lane was DNA marker and other lanes were PCR products.

The melting curves of samples were examined and the data generated from PCR was accepted only if the shape and melting temperature of the curve matched with that of our target genes. The experimental setups of melting curve in qRT-PCR were set to (1) denature all DNA PCR products at 95℃ for 5 seconds, (2) hold the samples at 65℃ for 1 minute and (3) increase the temperature gradually to 97℃ with continuous signal acquisition. Also the Ct values of controls (HK1007, HK1880, HK1908 and four serial dilutions of Control-R) were tried to match with the standard curve and checked whether the obtained Ct values fitted the standard curve or

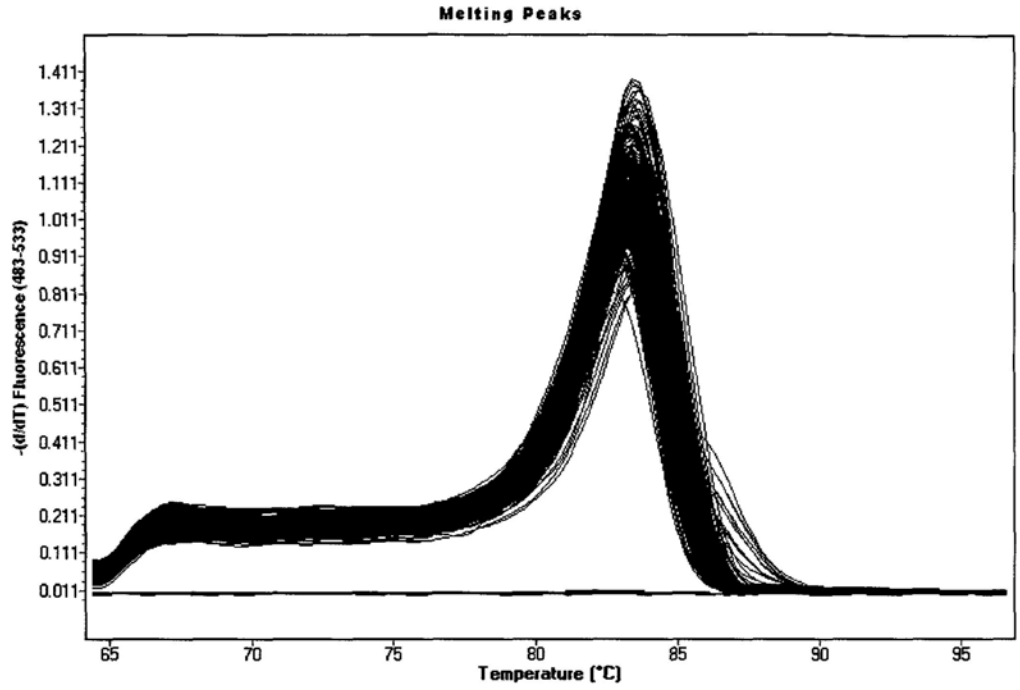

Figure 3.13 The melting curve of amplified telomere products

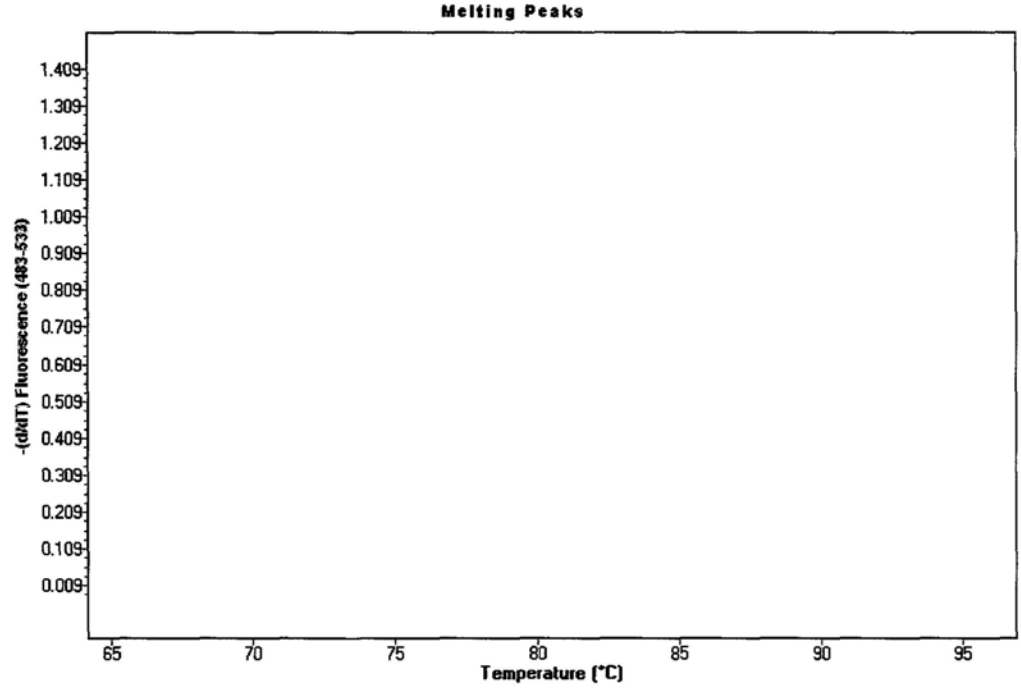

Figure 3.14 The melting curve of 36B4 PCR products

3.2.5 Calculation of Telomere Length

After qRT-PCR, the Ct values of telomere measurement assay and control assay of sample were obtained. The Ct values then required correction for PCR efficiency.

To calculate the efficiency, a curve of Ct values of the four serial diluted (10ng/μl, 5ng/μl, 2.5ng/μl and 1.25ng/μl) Control-R samples obtained from the PCR experiment versus the logarithm of the four diluted Control-R DNA concentrations was constructed. The PCR efficiency of the experiment could be calculated by the formula $10^{-(1/slope)}$ and the acceptable range was from 1.5 to 2.2.

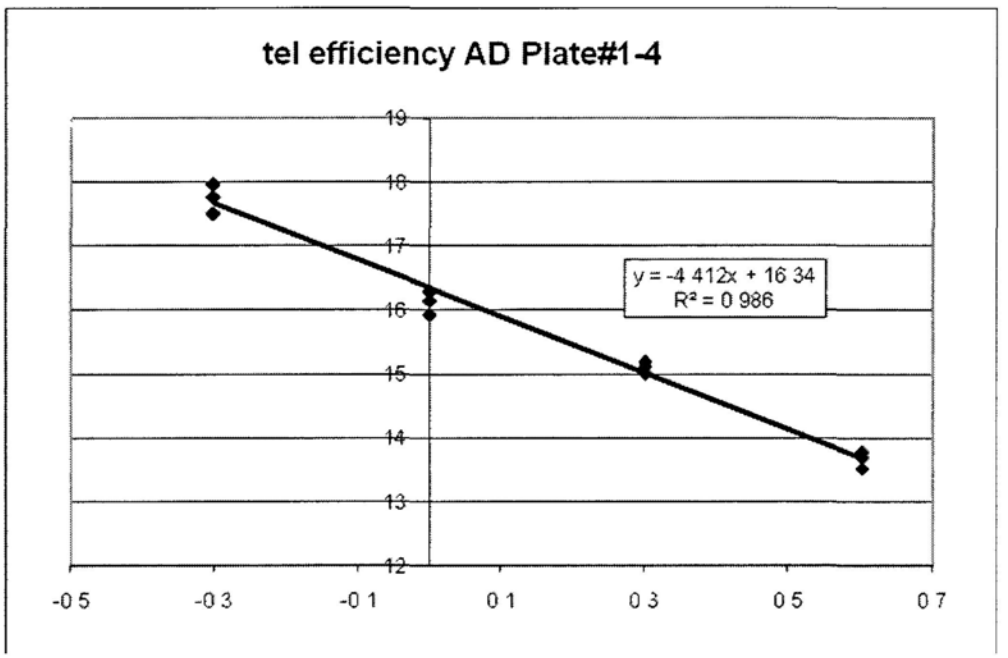

Figure 3.15 The curve (X-axis: Log of DNA concentration, Y-axis: Ct) constructed from telomere for PCR efficiency

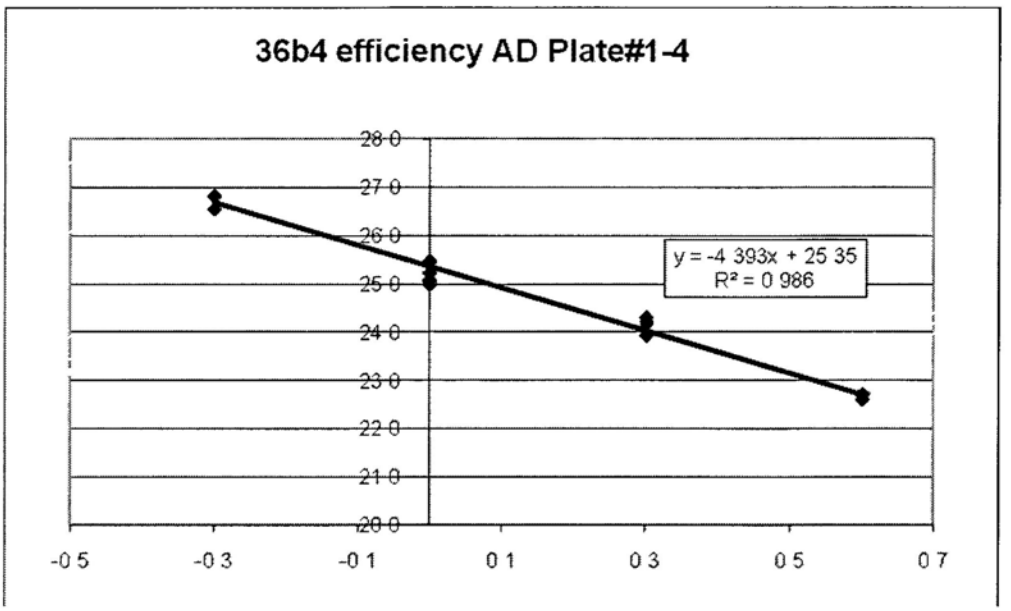

Figure 3.16 The curve (X-axis: Log of DNA concentration, Y-axis: Ct) constructed from 36B4 for PCR efficiency

The ratio of abundance of telomere repeats to copy number of 36B4 (T/S ratio) was positively correlated to the telomere length.

$$\text{T/S ratio of a single sample} = \frac{(\text{PCR efficiency of the T assay plate})^{C_t \text{ of sample in T assay}}}{(\text{PCR efficiency of the S assay plate})^{C_t \text{ of sample in S assay}}}$$

The T/S ratio was used to calculate the relative T/S ratio (ΔΔCt) by comparing the T/S ratio of sample to that of Control-R using the following equation.

$$\text{Relative T/S ratio } (\Delta\Delta C_t) = \frac{\text{T/S ratio of a single sample}}{\text{T/S ratio of universal reference (Control-R)}}$$

A calibration curve of the telomere lengths of the four controls (HK1007, HK1880, HK1908 and Control-R) measured previously by TRF analysis against ΔΔCt values was plotted with the data of the four controls measured in the qRT-PCR

experiment.

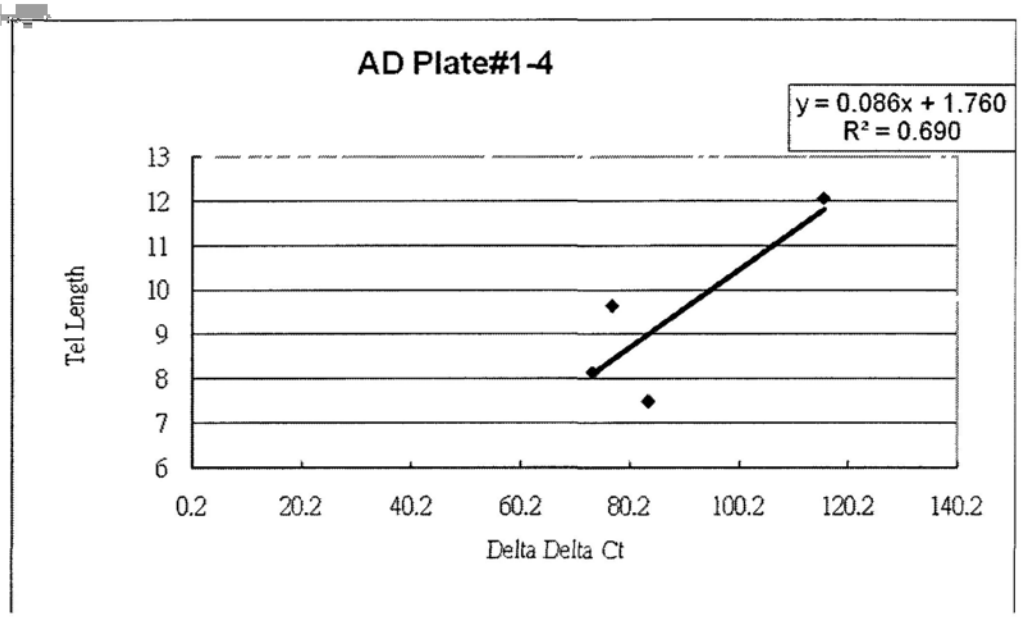

Figure3.17 The plot of telomere lengths obtained from TRF analysis versus medium ΔΔCt ratios of the four controls (HK1007, HK1880, HK1908 and Control-R) obtained from experiment.

The coefficient of determination (R^2) of the linear correlation between the telomere length and ΔΔCt was 0.69 and the value of R^2 above 0.64 (R=0.8) was accepted as the ΔΔCt values fell into acceptable of linear correlation.

3.2.6 Selection of Subjects with Quality Controlled qRT-PCR Data

All data obtained from qRT-PCR were required to be screened through several selection criteria before preceded to analysis. The first criterion was that the amplified PCR products should have melting curve with correct shape with no unknown peak. The second criterion was the PCR efficiency of the experiment should

fall in between 1.5 and 2.2 and the relative T/S ratio (ΔΔCt) of the four controls, which were placed in every 386-well PCR testing plate, should be not significantly varied from a linear relationship, i.e. R^2>0.64. (Figure 3.17).

The above criteria aimed to assure the qRT-PCR experiment worked properly and amplified the desired products successfully. After that, the relative T/S ratios were calculated and the highest and lowest 15% of data were removed as outliers. Samples without complete dataset were not included. After all screening procedures, there were 150 AD subjects and 261 non-demented subjects included in the analysis.

3.3 Telomere length association study in community dwelling volunteers.

The study was carried out by the School of Public Health of the CUHK and the major investigator was Prof. J. Woo. Subject recruitment and interview, blood collection and other experiments were done by her student Mr. Eddie Suen. Permission had been obtained to use part of the dataset for analyses in this thesis. The data of age, education level, MMSE score and relative T/S ratio (ΔΔCt) of 2000 male samples were obtained and further statistical analysis were carried out by me. 976 samples were used for analysis after selections which include screening out samples with telomere length in extreme 15% and without complete data.

Furthermore, subjects who scored at the lowest 25% percentile of MMSE were compared with subjects with higher scores. The cutoff point was found to be 23/24 in this sample. This selection of subjects with poor global cognitive performance also corresponded with the standard cutoff of cognitive impairments in Caucasian populations. However, the selection of cognitive impairment in the healthy aging sample is different from the local cutoff for clinical dementia as participants in the present sample was not classified as dementia in this exercise.

5 control subjects were chosen for standard curve construction using the methodology mentioned in section 3.2.3. The control samples included 3 elderly (HK1007, HK1880 and HK1908) and 2 young people (Control-J and Control-R).The relative T/S ratio of each sample was measured by qRT-PCR. The machine used was Roche Light Cycler 480 and the single copy control gene was 36B4. The primer sets for amplification of telomere and 36B4 were with same sequences stated in section 3.2.4. However the reaction mix and condition were different. Commercial kits Roche LightCycler 480 SYBR Green I Master were used for telomere and 36B4 reactions and the reaction setups were stated as below.

For telomere reaction:

1 95°C for 10 minutes

2 95°C for 5 seconds

3 56°C for 10 seconds

4 72°C for 60 seconds

5 Repeat steps 2 to 4 for 30 cycles

For 36B4 reaction:

1 95°C for 10 minutes

2 95°C for 5 seconds

3 58°C for 10 seconds

4 72°C for 40 seconds

5 Repeat steps 2 to 4 for 20 cycles

After collecting data from the qRT-PCR, data were subjected to some quality control steps. Firstly, the highest and lowest 5% Ct values were removed. Afterwards those samples with both of their telomere and 36B4 Ct values not excluded were further selected with those lied outside the first and third inter-quartile range were screened out.

After all selection procedures, there were 976 sample data left for further analysis like relative T/S ratio (ΔΔCt). The procedures for relative T/S calculation could be referred to sections 3.2.5 and 3.2.6.

3.4 Statistical Analysis

In the estimation of sample size needed for analysis, it is assumed that if the frequency of risk SNP allele was 15% and the allele could post a 2-fold disease onset risk, 200 AD and 200 normal subjects were required in order to provide a 80% power to detect allele with significantly impact (p-value < 0.05).

All descriptive and analytical statistics were performed by SPSS 17.0. Chi-square (χ^2) test was used to compare the cell cycle checkpoint genes SNP and diagnosis of AD. Mann-Whitney U tests were used to compare the mean of relative T/S ratio between AD and non-demented subjects. Pearson correlation and non-parametric Spearman's correlation were used to study the association. Linear regression was performed to identify the relationship between telomere lengths and global cognitive function as measured by Cantonese MMSE. The significant level was set to be 0.05 (two-sided).

CHAPTER 4. Results

This section was divided into three parts, i.e. (1) cell cycle checkpoint gene single nucleotide polymorphism association study, (2) association study of telomere lengths in a clinical sample of AD and (3) association study of telomere Length in community dwelling older men in a healthy aging study

4.1 Association study of Cell Cycle Checkpoint Gene polymorphisms and AD Association Studies

4.1.1. Demographic characteristics

Information from 701 participants was obtained for this study. Two hundred and fifty five were diagnosed as AD and four hundreds and forty-six were not clinically demented. Age and gender distribution was reported in the section 3.1.1 of Methodology.

4.1.2 Results of AD and cell cycle checkpoint gene SNPs association study

4.1.2.1 Retinoblastoma 1 gene (RB1)

For the 4 SNPs related to the RB1 gene, there was no significant association with the diagnosis of AD (chi square, $p>0.05$).

SNP #	p-value
rs9603847	0.998
rs1981434	0.541
rs990814	0.301
rs1573601	0.186

Table 4.1.1 Association between RB1 SNPs and AD

4.1.2.2 Cyclin-dependent kinase inhibitor 1A gene (CDKN1A)

For the 6 tag SNPs related to the CDKN1A gene, there was no significant association with AD (chi square, $p>0.05$).

SNP #	p-value
rs3176323	0.481
rs10947623	0.252
rs1801270	0.705

rs3176343	0.926
rs2395655	0.887
rs12207548	0.347

Table 4.1.2 Association between CDKN1A SNPs and AD

4.1.2.3 Cyclin-dependent kinase 5 activator 1 gene (CDK5R1)

For the 9 SNPs related to the CDK5R1 gene, there was no significant association with AD (Chi square, $p > 0.05$).

SNP #	**p-value**
rs2286246	0.179
rs2285428	0.322
rs4523973	0.288
rs731880	0.802
rs756785	0.950
rs756787	0.719
rs9333303	0.354

rs9889771	0.858
rs8069868	0.734

Table 4.1.3 Association between CDK5R1 SNPs and AD

4.1.2.4 Cyclin-dependent kinase 2-associated protein 1 gene (CDK2AP1)

For the SNP related to the CDK2AP1 gene, there was no significant association with AD (chi square, $p > 0.05$).

SNP #	**p-value**
rs3759114	0.529

Table 4.1.4 Association between CDK2AP1 SNPs and AD

4.1.2.5 Cyclin-dependent kinase inhibitor 2A gene (CDKN2A)

For the 5 tag SNPs related to the CDKN2A gene, there was no significant association with AD (Chi square, $p > 0.05$).

SNP #	**p-value**
rs2811712	0.201
rs3731257	0.492
rs2811710	0.649
rs3731217	0.546
rs3218020	0.105

Table 4.1.5 Association between CDKN2A SNPs and AD

4.1.2.6 Murine double minute 2 gene (MDM2)

For the 3 tag SNPs related to the MDM2 gene, there was no significant association with AD (Chi square, $p > 0.05$)

SNP #	**p-value**
rs2870820	0.337
rs3730581	0.701
rs1695147	0.782

Table 4.1.6 Association between MDM2 SNPs and AD

4.1.2.7 Tumor protein 53 gene (p53)

For the 3 tag SNPs related to the p53 gene, there was no significant association with AD (Chi square, $p > 0.05$).

SNP #	**p-value**
rs17882227	0.427
rs4559962	0.299
rs12951053	0.735

Table 4.1.7 Association between p53 SNPs and AD

4.1.2.8 Glycogen synthase kinase 3 beta gene (GSK3B)

For the 4 tag SNPs related to the GSK3B gene, there was no significant association with AD (Chi square, p> 0.05).

SNP #	p-value
rs10934503	0.902
rs16830594	0.671
rs2199503	0.782
rs13321782	0.408

Table 4.1.8 Association between GSK3B SNPs and AD

4.1.2.9 Cyclin-dependent kinase inhibitor 1B gene (CDKN1B)

For the 4 tag SNPs related to the CDKN1B gene, there was no association with AD (Chi square, p> 0.05).

SNP #	p-value
rs34330	0.189

rs34322	0.681
rs12229100	0.310
rs3759216	0.274

Table 4.1.9 Association between CDKN1B SNPs and AD

4.1.2.10 Transcription factor Dp-1 gene (TPDP1)

For the 4 tag SNPs related to the TPDP1 gene, there was no association with AD (Chi square, P> 0.05).

SNP #	**p-value**
rs4150715	0.830
rs4150735	0.958
rs9577581	0.798
rs12867405	0.877

Table 4.1.10 Association between TPDP1 SNPs and AD

4.1.11 Summary

The findings suggested that the cell cycle checkpoint gene polymorphisms were not associated with diagnosis of clinical AD.

4.2 Association study of Telomere Lengths and global cognitive function in a clinical sample of AD

4.2.1 Demographic characteristics

One hundred and fifty subjects satisfying diagnosis of AD and 261 non-demented elders were recruited in this association study. Two hundred and ninety eight were women and 113 were men. The mean age in years of women was 74.8(8.8) and 72.3(6.9) respectively. There was no significant different between men and women in term of age after T-test (p-value>0.05).

The average numbers of year of education received by the subjects were 5.3 years with S.D. 4.7 years. When the figures were divided into male and female groups, it was found out that men had received education for 8.0 years in average (S.D. 4.5 years) while women were just received around half of the average education years of men (mean=4.0 years, S.D.=4.3 years). After analyzing the data, there was no significant different in term of education years between men and women (p-value>0.05).

4.2.2 Telomere lengths

The telomere length was represented by its relative T/S ratio. Telomere length

was positively proportional to T/S ratio. The mean value of telomere length was 0.58 with S.D. 0.20. Figure 4.5 showed that the relative T/S ratio roughly followed a normal distribution.

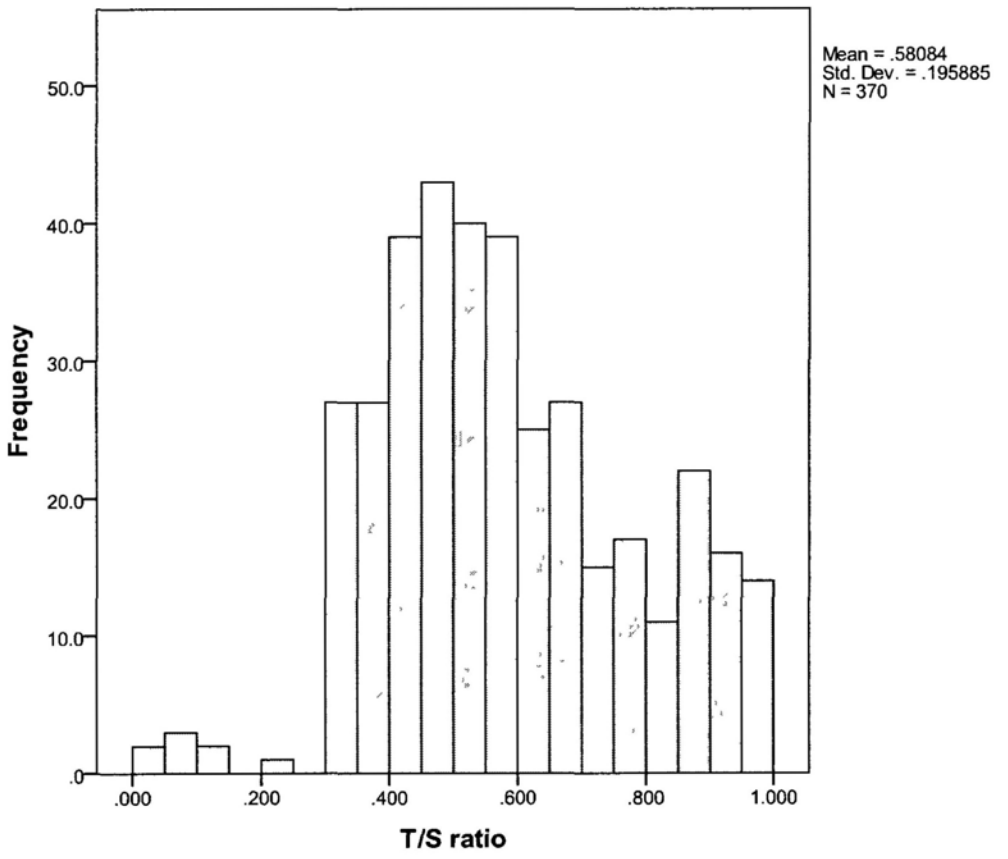

Figure 4.2 Distribution of T/S ratio

In women, the mean of T/S ratio was 0.57 (S.D. 0.20) while the mean was 0.61 (S.D. 0.19) in men. There was no significant different between male and female in term of telomere length (T-test, p-value>0.05).

4.2.3 Global Cognitive Function

Global cognitive function was assessed by the CMMSE. In women, the medium

CMMSE score was 25. In men, the medium CMMSE score was 27. There was significant different between men and women.

4.2.4 Association of age and telomere length

Telomere length was significantly associated with age (Spearman rho, $p < 0.001$).

		Relative T/S ratio
Age	Correlation Coefficient	-0.177
	Sig.	<0.001

Table 4.1 Correlation of age to telomere length represented by relative T/S ratio

4.2.5 Association between MMSE score with telomere lengths and demographic parameters

	Education years	Age	Relative T/S ratio
Correlation with CMMSE	0.12	-0.50	0.14
Significance (2-tailed)	0.12	0.001	0.05

Table 4.2 Association between CMMSE scores, educational attainment, age and relative T/S ratio.

The MMSE score was marginally associated with telomere length ($p=0.05$). After controlling for the effects of age by partial correlational analysis, there was no association between MMSE and T/S ratio (partial correlation, $p > 0.05$) (Table 4.3).

	Relative T/S ratio
Correlation with CMMSE	0.06
Significance (2-tailed)	0.42

Table 4.3 Correlation between MMSE and relative T/S ratio controlled for the effects of age

4.2.6 Telomere length and diagnostic of AD

Telomere lengths were significantly different between AD and non-demented elderly (Mann-whitney U, $z= 3.80$, $p <0.001$)

4.2.7 Regression analysis for association of telomere length and MMSE

The strength of association between MMSE and T/S ratio was investigated using linear regression model and the association was significant (p-value=0.05). The regression model showed that the adjusted R^2 value of 0.015, inferring that the telomere length only accounts for 1.5% of total variance for CMMSE.

Logistic regression analysis was carried out to evaluate the relationships between clinical diagnosis of AD, MMSE scores, age and T/S ratio. Telomere length was not associated with diagnosis of AD (p =0.45), while age and MMSE score were strongly correlated with p-value 0.002 (Nagelkerke R^2=0.78).

4.3 Association Study of Telomere Length in community dwelling older men in a healthy aging study

4.3.1 Demographic characteristics

Nine hundred and seventy six men who participated in the community study for healthy aging were assessed for telomere length and association with global cognitive function. The age (in years) of the participants ranged from 65 to 91, with a mean age of 72.8 (S.D. 5.0 years) (Figure 4.3).

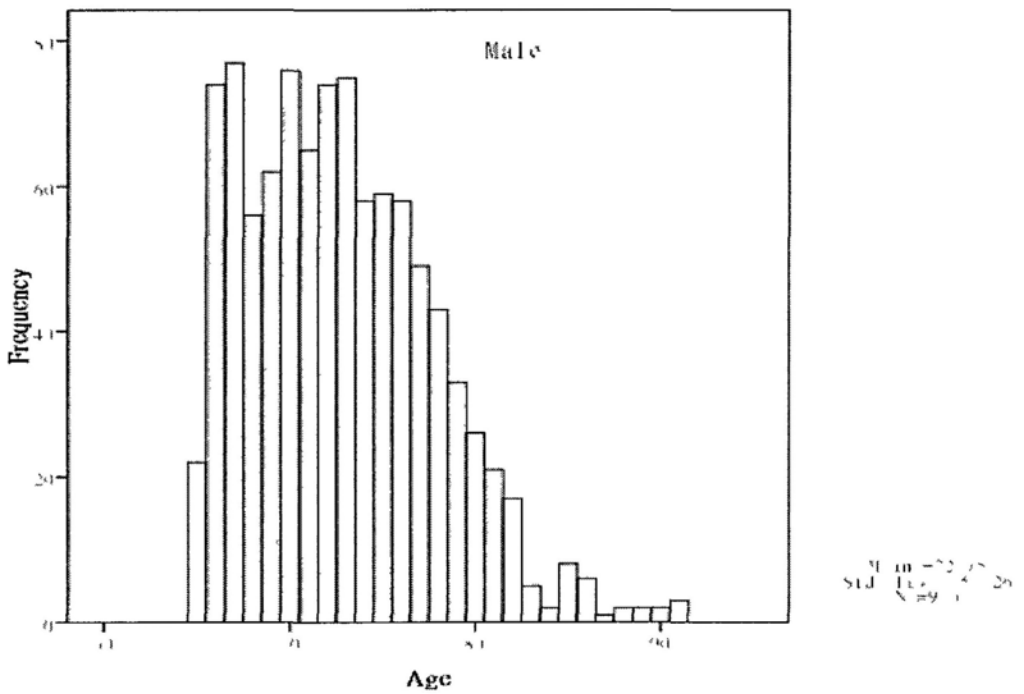

Figure 4.3 Frequency distribution of age.

Education level was categorized into 5 groups. Group 1 represented the subject received least education (no education) and group 5 meant highest education level (tertiary education and above). Thirty-nine percent of subjects were belonged to the Group 1 and 2 while only 12.7% received tertiary education or above.

4.3.2 Global Cognitive Function

Global Cognitive Function was estimated by the Cantonese version of MMSE. The range of score was from 10 to 30 and the medium score was 27.

4.3.3 Telomere lengths

The relative T/S ratio was used to represent the telomere length. Longer telomere is reflected by a higher relative T/S ratio. The range of T/S ratio was between 0.1 and 1.1, and the mean value was 0.62. The standard derivation of the ratio was 0.18. The normal distribution of the ratio was depicted in Figure 4.4.

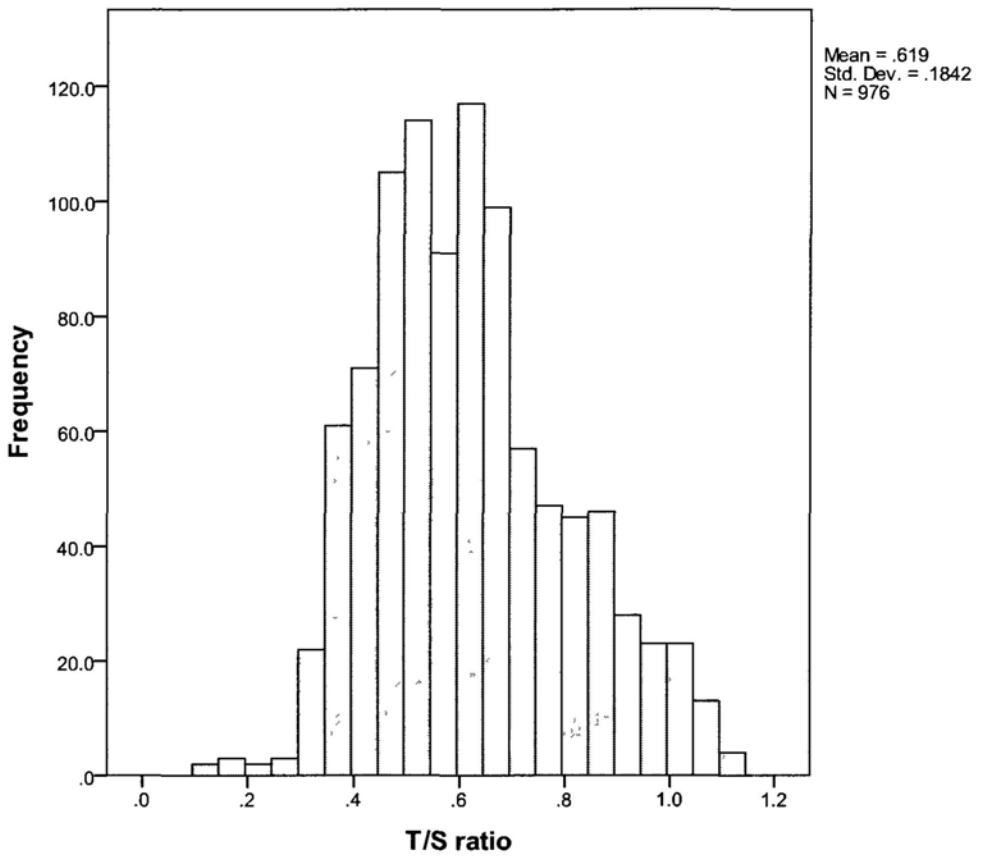

Figure 4.4 Distribution pattern of the relative T/S ratio.

4.3.4 Correlations between relative T/S ratio, age, education and MMSE scores

Age was found to be correlated with the relative T/S ratio (p-value=0.01). Education level and MMSE score were not associated with telomere lengths (Spearman Rho, p>0.05).

	Age	Education Level	MMSE
Correlation Coefficient with T/S ratio	-0.080*	-0.037	0.037
Sig. (2-tailed)	0.012	0.247	0.244

Table 4.4 Correlation of the relative T/S ratio with demographic parameters.

To further evaluate if relative T/S ratio was associated with subjects having

different global cognitive abilities, subjects who scored at the lower 25% (quartile) of CMMSE were grouped and compared with subjects who scored higher than the lowest quartile. The lower quartile cutoff score was 24. For subjects with CMMSE scores lower than or equal to 24, there were difference in relative T/S ratio compared the group who scored above 24 (Mann-Whitney U test, z=2.06, p=0.04) (Table 4.5).

CMMSE score	Number	Mean	S.D.	Z-score	p-value
less or equal to 24	177	0.60	0.19	-2.06	0.039
higher than 24	799	0.62	0.18		

Table 4.5 T/S ratios of the samples with CMMSE higher than 24 and other remaining group with low score. Mann-Whitney test for T/S ratio comparison between two groups.

However if the effect of age was controlled, there was no association found between telomere length and CMMSE score (p-value=0.31).

CHAPTER 5. Discussion

5.1.1 Association study of cell cycle checkpoint gene polymorphisms and AD

With reference to the results of SNP genotyping and AD association studies presented in section 4.1, SNPs of the 10 chosen genes, i.e. RB1, CDKN1A, CDK5R1, CDK2AP1, CDKN2A, MDM2, p53, GSK3B, CDKN1B and TPDP1 genes, were not associated with AD. Our findings suggested that cell cycle checkpoint gene polymorphisms are probably not significantly and directly implicated in the pathogenesis of AD, thus are unlikely to be useful biomarkers for the disease.

Although these genes are playing important roles in G1 and G2 checkpoints, they did not show up to be a critical part in the pathogenesis of AD. Most diseases related to cell cycle gene mutations present with earlier onset than late-onset sporadic AD, the target disease of my project. Polymorphisms of CDKN2A and E2F2 genes contribute significantly to the pathogenesis of early onset colorectal cancer; familial early onset cardiovascular diseases may be associated with mutations of genes regulating cyclin-dependent kinases (CDKs). As normal functioning of cell cycle checkpoints are important determinants of cellular survival and apoptosis, abnormalities in cell cycle genes would demonstrate devastating effects. It is thus reasonable to expect that for a disease which is late onset, important cell cycle

checkpoints may not be key determining factors. For late onset sporadic AD, a multi-factorial model is likely to be of higher face validity. The findings of lack of association between the cell cycle checkpoint genes' polymorphisms were consistent with this model.

While we failed to identify any significant association between SNPs of cell cycle checkpoint genes with AD, search for other high risk genes in downstream pathways for neurodegeneration, e.g. inflammation and oxidative stress, should be continued. The significance of genetic predisposition in affecting disease evolution should still have potential for development of a panel of valid, accessible and low cost blood biomarker for this disorder.

5.1.2 Education level variation between male and female

In section 4.2.1 the education level between male and female is significant. The different is due to unbalanced education opportunities that favor men to get enrolled into school. As the result of the study showed that education is not correlated to telomere length (Section 4.2.5) and MMSE (Section 4.3.4), the samples should still be useful to answer the research hypothesis.

5.1.3 Association between telomere lengths and global cognition ability

As outlined in the background, there is an upsurge of research interests about the clinical significance of telomere lengths in aging and cancer research. As an index to reflect cellular senescence, it would be of interest to explore if telomere lengths are associated with AD, the commonest neurodegenerative disorder of late life. In this project, we attempted to evaluate hypothesis from two independent groups of participants. The first group comprised of community dwelling male volunteers who were recruited for a project about healthy aging. The second sample was recruited from a clinic population and volunteers from social centers. It is hoped that the two samples would help to cross-validate the observations and provide information as to the association across the whole severity spectrum of cognitive function.

5.1.3.1 Age and telomere length

Both datasets gathered from community dwelling and clinical AD samples demonstrated that age correlated negatively with telomere lengths (Sections 4.2.4 and 4.3.4). The telomere contained repeated sequences of TTAGGG and a short section of it is lost during every round of cell replication. As a result of that, the telomere will be shortened during aging and our findings supported this hypothesis.

5.1.3.2 Education and telomere lengths

As stated in the section 4.2.5 and 4.3.4, educational attainment was not significantly associated with telomere length. Education is a well-known protective factor for clinical AD. A high educational attainment helped to offer better cognitive reserve and provide a person with higher compensatory capacity to resist the effects of neurodegeneration in dementia. As telomere length shortening was a physiological index for reducing cellular viability, it is reflective of primary disease process directly implicated in AD. Education may play a protective role in the clinical manifestations of AD by enhancing secondary strategies such as enriched neural networking and increase synaptogenesis. If the factor of education does not act through direct influence on the core pathogenic pathway, it should not have any association with markers that reflect the primary processes. Our negative findings may be interpreted as a direct lack of association between education and telomere length. It could also be considered as a indirect inference that the protective effect of education does not take place through affecting primary disease mechanism of AD, but are through effects on other mechanisms.

5.1.4 Global Cognitive function and telomere lengths

The telomere length (represented by relative T/S ratio) is showed not to be associated with CMMSE scores in the healthy aging study (Section 4.3.4). As the

sample comprised of mostly healthy older men, the ceiling effects of CMMSE scores may not be sensitive enough to capture subtle changes associated with persons having suboptimal cognitive performance. For the subjects with lowest 25% MMSE score in the health aging study and those subjects diagnosed as AD in the clinical sample, shorter telomere lengths were found. However the association was much weakened to an insignificant level if the effect of age was controlled. The findings suggested that the shortening of telomere could affect cognitive function but the effect was much less prominent when compared to that of age. It was believed that the telomere length shortening may represent secondary physiological changes associated with late clinical impairments of AD. As a result, the telomere length may not sensitive enough to diagnose pre-clinical AD or mild dementia, as telomere attrition is a later biomarker for cumulative oxidative stress and inflammation (150-152). When the significance of telomere lengths as a factor contributing to AD diagnosis was further explored by regression analyses, the overall effect size of was small (1.5%) and did not suggest a significant impact of telomere lengths accounting for AD diagnosis. The results echoed that AD is a multi-factorial disorder.

5.2 Limitations

5.2.1 Sample characteristics

While we tried to achieve a sizeable clinical sample for our SNPs and telomere studies (150 subjects with AD), the small effect sizes of the factors under studied precluded definitive conclusions. The gender and age distribution were not fully matched between the AD and non-demented groups. As age and gender were factors associated with the AD, this limitation may have confounded interpretations of the results. For further studies, it would helpful if more strict matching of demographic characteristics could be achieved in subject recruitment.

5.2.2 Scope of interview parameters

In the study, there were limited clinical parameters included, i.e. age, sex, MMSE and clinical diagnosis of AD. It will be better if we can expand the scope of interview to include more parameters exploring lifestyles and physical health status. This would help to evaluate the association controlling for other potential confounders, which was especially relevant for the telomere study.

5.2.3 Diagnostic biomarkers

Due to limited resources, there was no adjuvant biomarker such as neuroimaging measures included in the analysis. If neuroimaging and other biomarkers were included in the analysis, a more delicate profile of biomarker panel

could be explored for its potential to specify staging of AD.

5.2.4 Experimental conditions

In my project there were two sets of data included. One was dataset obtained from the CUHK School of Public Health community dwelling male volunteers for the health aging study and the other was clinical AD sample. As the experimental conditions, like duration of DNA storage, methods in DNA preparations and quantitative real-time PCR setups, differed and these variations may affect the amplification kinetics of targets and control gene which may cause differences in PCR efficiencies, the two datasets were not merged into one for analysis. While the two samples might serve as cross-validation for each other, it limits the effort to expand the sample size.

5.2.5 Follow-up data

There is no follow-up data available for disease progression analysis. Prospective data would provide valuable information in understanding the prognostic implications of telomere length attrition in persons with preclinical or asymptomatic AD.

5.3 Potential Clinical Implications of Findings

1. Telomere length may not be able to serve as a standalone bio-marker for clinical diagnosis of AD as it does not correlate with the MMSE in majority population and people with less severe psychiatric problem.
2. In clinical trials for clinical AD, telomere lengths may serve as a potential biomarker for stratification of risk. As telomere lengths may reflect underlying physiological function related to neurodegeneration, it may be interesting to evaluate if subjects having shorter telomere lengths would be less responsible to treatments with AD. It can be use as an adjuvant marker for selection of subjects into clinical trials. However, it must be used together with more sensitive and specific markers as the effect size contributing to clinical AD diagnosis was very small (1.5%).

5.4 Further work.

In the prospective study, more subjects should be recruited with special attention to matching of age and sex groups. Enrichment of the clinical and cognitive assessment should be attempted with includsion of other adjuvant biomarkers like CSF p-tau and amyloid level, neuroimaging and other genetic predisposition factor like ApoE genotype.

Instead of one-time interview and blood sampling, serial assessments should be conducted. This would allow us to have a monitoring of the expression patterns during the progression of AD and maybe helpful for further development of a panel of biomarkers to distinguish different stage of AD.

CHAPTER 6. Conclusions

The results of my study suggested that cell cycle checkpoint genes may not be useful markers for AD diagnosis.

Telomere length variations were associated with lower global cognitive function and diagnosis of clinical AD. However, the effect sizes were small and the interactions may be confound by age, a major determinant for cognitive function. Telomere length shortening may not suitable as a standalone diagnostic biomarker for AD, further studies should be conducted to examine its usefulness as an adjuvant biomarker for risk stratification of AD intervention trials.

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