

Genetic risk factors and candidate biomarkers for Alzheimer's disease

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TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Pathogenesis of AD
4. Genetic of AD
5. Biochemical markers
6. Conclusive remarks
7. References

1. ABSTRACT

Alzheimer's disease is a multifactorial and progressive neurodegenerative disease, extremely diffused and with an increasing prevalence worldwide. There is an urgent need for biomarkers to diagnose AD early in its course. Furthermore, accurate biomarkers would be able to determine the clinical efficacy of novel neuroprotective strategies. Although the heritability of late-onset AD is high, our knowledge of the underlying putative susceptibility genes remains incomplete and the only unequivocally established late-onset AD gene is *APOE*. Nevertheless a number of susceptibility loci seems to influence the pathogenesis of AD, and variations in numerous genes have been considered to be important in the risk for AD. Many advances have been made in identifying biochemical indices of brain dysfunction, measured in body fluids such as cerebrospinal fluid and plasma, with different methodological approaches. Although these biomarkers are promising, none of them can predict AD with 100% confidence to date. This review will elaborate on the available selection of genetic and biochemical biomarkers for AD, with a particular reference to those linked to inflammation and oxidative stress.

2. INTRODUCTION

Alzheimer's disease (AD) is a heterogeneous and progressive neurodegenerative disease which in Western society mainly accounts for clinical dementia. Neurodegeneration in AD appears to be multifactorial, whereby several biochemical processes operate sequentially and/or in parallel. Neuro-pathological hallmarks are senile plaques, resulting from the accumulation of several proteins and an inflammatory reaction around deposits of amyloid, a fibrillar protein, Abeta, product of cleavage of a much larger protein, the beta-amyloid precursor protein (APP) and neurofibrillary tangles. Amyloid deposition, due to the accumulation of Abeta peptide, is the main pathogenetic mechanism. It is quite clear that similar or identical pathological lesions can be the consequence of multiple environmental and genetic susceptibility factors, and thus, the initial causative biological processes may differ between the affected individuals. The pathological process characteristic of AD begins decades before the first symptoms of brain failure, thus making it difficult to reliably identify pathology based on the clinical phenotype alone. The increasing prevalence of AD motivate the drive to develop diagnostic biomarkers

to reliably identify the pathology at an early stage. Although many neurodegenerative diseases cannot be cured at the present time, there are often symptomatic treatments available and new drugs are emerging to forestall and/or reverse the onset and/or progress of the diseases (1, 2). Thus, an early diagnosis will at least assist in the better management of patient care. Biomarkers for AD may also help to identify subclasses of the disorders, monitoring of disease progression and treatment (3). Advances have been made in neuroimaging techniques that assess regional structure, function and biochemistry of the brain, as well as in identifying biochemical indices of brain dysfunction, measured in body fluids such as cerebrospinal fluid (CSF) and plasma (4). This review will elaborate on the available selection of genetic and biochemical biomarkers for AD, with a particular reference to those linked to inflammation and oxidative stress, since the review is mostly focused on data from author laboratories.

3. PATHOGENESIS OF AD

Amongst the existing entities of dementia spectrums, Alzheimer's disease and dementia with vascular component are the most prevalent forms of dementia. These disorders have common and unique molecular pathological characteristics that result in serious reductions in nervous-system functionality (5). AD, the most common cause of dementia, accounts for 50 to 70 percent of dementia cases (6). It is a severe neurodegenerative disorder characterized by progressive memory and cognitive impairment. The multiple pathogenic events in AD can be classified as primary events (genetic factors, neuronal apoptosis), secondary events (beta-amyloid (A β) deposition in senile plaques and brain vessels, neurofibrillary tangles due to hyperphosphorylation of tau proteins, synaptic loss), tertiary events (neurotransmitter deficits, neurotrophic alterations, neuroimmune dysfunction, neuroinflammatory reactions) and quaternary events (excitotoxic reactions, calcium homeostasis miscarriage, free radical formation, primary and/or reactive cerebrovascular dysfunction) (7). Under physiological conditions, APP is processed by the non-amyloidogenic pathway, where cleavage by α -secretase releases a soluble fragment. In AD, this process is significantly altered, where increased amount of APP is cleaved by other endo-proteases such as β - and γ -secretase, generating highly amyloidogenic β -amyloid protein molecules of 40-42 amino acid residues. Soluble β -amyloid protein rapidly aggregates into fibrils triggering the misfolding of other A β species. In vitro studies have shown that extracellular fibrillar A β peptides induce apoptosis in cultured neurons (8). The amyloid cascade hypothesis is the central hypothesis for the cause of AD, which states that the initiating event in AD is an imbalance between the production and clearance of A β in the brain (9). Another neuropathological hallmark of AD is the appearance of neurofibrillary tangles that consist of a hyperphosphorylated form of the microtubule-stabilizing protein tau, often conjugated with ubiquitin. The abnormal hyperphosphorylation of tau makes it resistant to proteolysis and this might lead to several-fold increase in the levels of tau in AD. The hyperphosphorylated tau

causes sequestration of normal tau and other microtubule-associated proteins, leading to inhibition and disruption of microtubules and impaired axonal transport (10). Tau also becomes prone to aggregation leading to formation of intracellular neurofibrillary tangles, compromising neuronal and synaptic function. A recent study has suggested a link between amyloid and neurofibrillary tangles, whereby A β exposure triggers caspase cleavage of tau, which in turn promotes the assembly of tau into pathological filaments (11). Several other hypotheses have been proposed to explain the pathogenesis of AD, including abnormalities in proteins regulating the cell cycle, inflammatory mechanisms, oxidative stress, and mitochondrial dysfunction with disruption in neuronal energy metabolism. In particular generation of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) associated with mitochondrial dysfunction have been demonstrated to play an essential role in the pathogenesis of this disease (12).

4. GENETIC OF ALZHEIMER'S DISEASE

Although the complete etiopathogenesis of AD still remains unclear, genetic studies over the past two decades have provided valuable insights into this complex and heterogeneous disorder. Twin and family studies have shown that certain genes contribute to the development of AD, especially with respect to the age at which the disease manifests, and more recently, the development of non-cognitive symptomatology (13). Early onset familial AD is a very rare autosomal dominant disorder caused by highly penetrant mutations in APP and presenilin genes, both linked to A β metabolism. Around twelve different mutations have been identified in APP gene at the level of α -, β -, or γ -secretase cleavage sites, which can lead to alteration in the normal proteolysis of amyloid precursor protein. Similarly, more than fifty missense mutations of the presenilin-1 gene (PS1) are associated with familial AD; several mutations of presenilin-2 gene (PS2) are associated with rare cases of early onset familial AD (14). These mutations of APP, PS1 and PS2 may share a common pathogenetic mechanism leading to accumulation of β -amyloid protein as a result of abnormal amyloid precursor protein metabolism. In contrast, sporadic AD is a very common disorder. Although the heritability of late-onset AD is high, our knowledge of the underlying putative susceptibility genes remains incomplete. The only unequivocally established late-onset AD gene is *APOE* (OMIM 107741), encoding apolipoprotein E, a protein involved in the transport of cholesterol. Three apoE gene alleles are described (epsilon 2, epsilon 3, and epsilon 4). A growing volume of evidence has reported an association of apoE epsilon 4, late-onset familial, and sporadic AD (15).

ϵ 4 allele increases the risk for AD by 4- to 15-fold in a dosedependent manner, and epsilon 2 has shown a protective effect. As many as 40 to 50% of AD patients possess ϵ 4 allele compared to 15 to 25% of controls. Subjects homozygous for epsilon 4 are reported to have a 6- to 8-fold increased risk of developing AD compared to the risk of heterozygotic subjects (increased by 2- to 4-

fold). Moreover, APOE epsilon 4 may influence AD pathology by interacting with APP metabolism and β -amyloid protein accumulation, enhancing hyperphosphorylation of tau protein and neurofibrillary tangle formation, reducing choline acetyltransferase activity, increasing oxidative processes, modifying inflammation-related neuroimmunotrophic activity and glial activation, altering lipid metabolism, lipid transport and membrane biosynthesis in sprouting and synaptic remodelling, and inducing neuronal apoptosis. To date, no other late onset AD gene has been conclusively proven, nevertheless a number of susceptibility loci seems to influence the pathogenesis of AD. Several studies have reported susceptibility loci on chromosome 1, 2, 5, 9, 10, 12, 14, 18, 19 (close to ApoE), and 21 (close to the APP gene) (16, 17). Genomewide association analyses of AD reported evidence of an association between variants in GRB2-associated binding protein 2 (Gab2) (OMIM 606203) on chromosome 11q14 and AD risk (18). Gab2 is a member of a family of evolutionarily highly conserved scaffolding and adapter proteins that are involved in multiple signalling pathways and particularly in the transduction of cytokine and growth receptor signalling (19). Gab2 is ubiquitously expressed but is found at high levels in white blood cells, prefrontal cortex, and hypothalamus (20). Changes in Gab2 expression could potentially affect Gsk3-dependent phosphorylation of tau and the formation of neurofibrillary tangles (21). Moreover, growth factor receptor-bound protein 2, which binds Gab2, has been reported to bind tau, APP, and presenilin 1 and 2, giving sense for the involvement of this gene in the pathogenesis of AD (22). Furthermore *GAB2* effect sizes are among the strongest and most significant observed in any putative disease gene after *APOE* in the field of AD. Variations in inflammation and apoptosis genes, such as HLA-A2, interleukins (IL1A, IL1B, and IL6) and tumor necrosis factor-alpha (TNF) have also been considered to be important in the risk for AD (23, 24). Other polymorphisms that may also be associated with AD are linked to the angiotensin-converting enzyme, Cystatin C, tau genes, estrogen receptor and 5-lipoxygenase enzyme. Genes involved in the neurodevelopmental process have also been considered good candidates to confer susceptibility to AD. All these genetic factors may interact in unknown genetic networks leading to a cascade of pathogenic events characterized by abnormal protein folding, with subsequent accumulation of abnormal proteins, ubiquitin-proteasome system dysfunction, excitotoxic reactions, oxidative stress, mitochondrial injury, synaptic failure, altered metal homeostasis, axonal and dendritic transport dysfunction and chaperone misoperation. In particular, cytokine gene polymorphisms have been claimed to play a key role in pathophysiology of AD. Several studies report associations between IL-1beta polymorphisms and AD, but findings from different studies are controversial. We have recently performed a meta-analysis to verify the correlation between the single nucleotide polymorphisms (SNPs) of the IL-1beta, at sites -511 and +3953, and AD (25). The results support an association between the TT genotype of IL-1beta +3953 SNP and AD, and suggest a possible association of the -511 TT genotype.

5. BIOCHEMICAL MARKERS

The biochemical markers of AD can be classified as primary (specific), such as Abeta or Tau, or secondary to the disease, or they can simply be epiphenomenal in nature. A wide variety of different proteins such as inflammatory markers, markers of oxidative stress, apolipoproteins, and markers of neuronal degeneration in blood and cerebrospinal fluid have been examined. The cerebrospinal fluid has been the principal focus of research for diagnostic markers in AD pathology due to its direct contact with the extracellular space of the brain (26), and the quantification of tau and Abeta in the CSF represent the most intensively studied biomarkers of AD (27). Approximately 80% of patients who meet clinical criteria for AD have elevated levels of CSF tau. The relationship between high levels of CSF tau and a pathologic diagnosis of AD has been confirmed also in autopsy studies (28). High CSF total tau (t-tau) has been proposed as a marker able to discriminate between memory-impaired individuals that later progressed to AD, and those that did not convert (29). Other authors have shown that the absolute level of CSF tau in patients with AD did not correlate with the severity or duration of the dementia (30). Another line of recent evidence suggests that CSF phosphotau (tau protein phosphorylated at threonine 231) declines during the natural course of AD. In this study the authors demonstrated that CSF phosphotau concentration, but not total tau, decreased over time in AD, independent of age. Rate of change was inversely correlated to cognitive decline, suggesting that CSF phosphotau may have the potential to track AD progression.

A β (1-42) is especially prone to fibrillization and disproportionately accumulates in extracellular lesions in AD brains, and most studies showed that Abeta 1-42 concentrations are lower in the CSF of AD patients. Decrease in CSF Abeta 1-42 in AD is probably the most consistent BM finding, and has been hypothesized to reflect a deposition of the peptide in senile plaques, with lower levels diffusing to the CSF. Abeta 1-42 alone showed a sensitivity of 78%, and a specificity of 81%, in distinguishing AD patients from elderly controls (32). However, studies correlating CSF Abeta 1-42 protein concentrations with cognitive performance in AD were, in part, contradictory (33) and the potential value of Abeta 1-42 protein during the course of AD progression should be further evaluated. Plasma Abeta 1-40 and Abeta 1-42 levels did not correlate with the disease (34), and results from different studies are often conflicting (35). A recent research conducted in presymptomatic familial AD persons indicated that plasma Abeta 1-42 is elevated in familial AD mutations carriers and that this level may decrease with the cognitive decline of disease progression prior to the development of dementia (36). Looking for secondary markers, advanced proteomic approach has provided numerous potential biomarkers to differentiate AD from non-AD with high sensitivity and specificity (for a review see 37). Using LC-MS platform, a recent research has revealed more than 100 candidate markers, including brain-derived neurotrophic factor (BDNF), interleukin (IL)-8, vitamin D binding protein (VDBP), apolipoprotein (apo)

A β , and apoE, as potential CSF biomarkers for AD (38). In a more recent SELDI-TOF-MS study, 15 potential biomarkers were identified and a panel of five markers (Cystatin C, truncated Cystatin C, A β 1–40, C3a anaphylatoxin des-Arg and a 4.0 kDa protein) together with total tau and A β 1–42 analysis could distinguish AD from healthy control individuals with high sensitivity and specificity (39). Finally, because blood is more easily accessible than CSF, a search is also underway for useful plasma biomarkers in AD. A recent research has demonstrated increased concentrations of complement factor H and alpha-2-macroglobulin (40), while another study identified four potential biomarker peaks using the serumalbumin-bound fraction from AD and control subjects (41).

It is widely believed that A β deposition in the brain starts an immune reaction leading to the development of the local chronic reaction typical of AD. Recently, our group has reported data on immune-inflammatory parameters evaluated in PBMC obtained from AD patients (42). We showed no changes in lymphocytes subsets with the exclusion of B cells that are reduced in AD subjects. The study of B cell naïve/memory compartment shows a reduction of DN (IgD-CD27-) B cells in AD patients compared with age-matched healthy controls. Inflammatory cytokines IL-1 β , IL-6, IFN- γ , TNF- α , chemokines MIP-1 β and RANTES as well as chemokines receptors CCR2, CCR3 and CCR5, are up-regulated in AD patients after *in vitro* stimulation with amyloid- β (rA β 42) peptide.

Also CD36, a scavenger receptor, is over-expressed in monocytes of AD patients. All together these data confirm the involvement of systemic immunity in AD and suggest to continue these kind of study to obtain biomarkers useful in the monitoring the effectiveness of therapeutics.

Fibroblasts of sporadic AD patients represent another important starting point in the research for novel biomarkers because of their various abnormalities in metabolic and biochemical processes, which reflect some of the events in the AD brain (43). A recent research has demonstrated an abnormal response of AD fibroblasts to an acute oxidative injury; in particular, fibroblasts from AD patients were found to be less vulnerable to the oxidative injury induced by H₂O₂ in comparison with fibroblasts from non-AD subjects (44). The tumor suppressor and transcription factor p53 plays a pivotal function in neuronal apoptosis triggered by oxidative stress. On the basis of immunoprecipitation studies with conformation-specific p53 antibodies, which discriminated folded vs. unfolded p53 tertiary structure, it has been found that in fibroblasts from AD patients a significant amount of total p53 assumes an unfolded tertiary structure in comparison with fibroblasts from control elderly subjects (45). In addition and most importantly, another research (46) has proposed a rapid, easy and quantitative flow cytometric approach for the discrimination of conformational mutant p53-bearing cells from AD patients compared to non-AD controls, using small volumes of blood. Using this technique, they

processed 75 AD, 66 controls, 15 subjects affected by another neuroinflammatory disease, Parkinson's disease and 3 subjects affected with other types of dementia (2 vascular dementia; 1 progressive supranuclear palsy) and confirmed the previous findings: AD subjects expressed higher levels of unfolded p53 in comparison with controls and subjects with other neurological diseases. Within this specific age interval (< 70 years), a comparison of the sensitivity and specificity values of this approach with those published in several studies, which evaluated the diagnostic power of CSF markers for AD (Total-tau, Phosphotau and A β 1–42), reveal that p53 measurement is more sensitive (90% compared to respectively 81.4%, 81.3% and 85.9%), but less specific (77% compared to respectively 91.5%, 91.2% and 88.5%). On the whole, these data strongly suggest that the measurement of conformational altered p53 in blood cells has a high ability to discriminate AD cases from normal ageing, Parkinson's disease and other dementias (47).

Additionally biomarkers could also be identified in human brain tissues. It has been observed that in AD frontal cortex autopsies protein kinase C (PKC) translocation is blunted when compared to age-matched controls (48) and this can be correlated with a defective expression of RACK1 levels (49), underscoring an alteration in PKC signal transduction in human brain under conditions of memory impairment. From a pharmacological perspective in animal models of AD, activation of brain PKC (such as with bryostatin-1) can influence beta amyloid deposition and clearance (50) and counteract behavioral deficits. The possibility to utilize PKC as a potential AD biomarker is encouraged by the fact that changes in PKC signal transduction are reported also in peripheral tissues such as fibroblasts and lymphocytes from AD patients (for a review see 51). Studies have applied proteomic technologies to characterize specific proteins in AD brain, for example, using redox proteomics (a branch of proteomics that identifies oxidatively modified proteins) a number of proteins that are oxidatively modified in AD brain have been identified (52, 53). Cortical and hippocampal oxidative stress is a very early event in the pathogenesis of sporadic AD and correlates with the development of specific cognitive deficits in this condition. Heat shock proteins have been regarded as cytoprotectants that protect brain cells from damage encountered following cerebral ischemia or during the progression of neurodegenerative diseases. Heme oxygenase-1 (HO-1) is a 32-kDa stress protein that catalyzes the degradation of heme to biliverdin. Increased expression of HO-1 is a common feature in a number of neurodegenerative diseases (54). The HO-1 gene is redox regulated and its activation could represent a protective system potentially active against brain oxidative injury (55). HO-1 has been also proposed as a potential biomarker for AD, and its expression in AD patients brain is significantly increased (56). Interestingly, the spatial distribution of HO-1 expression in diseased brain is essentially identical to that of pathological expression of tau (57). HO-1 immunoreactivity is greatly increased in neurons and astrocytes of the hippocampus and cerebral cortex of individuals with AD and colocalizes to senile plaques and

neurofibrillary tangles. HO-1 is thought to down regulate the production of tau and recently HO-1 polymorphisms have been considered as a possible responsible for susceptibility to AD (58). Plasma and CSF HO-1 protein and lymphocyte HO-1 mRNA levels have been demonstrated to decrease in subjects with sporadic AD, compared to normal elderly controls and non AD neurologic patients (59). In particular the sensitivity and specificity of lymphocyte HO-1 mRNA measurement for diagnosis of early sporadic AD presented in this study are 88% and 75% (60).

6. CONCLUSIVE REMARKS

Several experimental methods have been used to identify single biomarkers in early disease stage of AD. Although these biomarkers are promising, none of them can predict AD with 100% confidence nor provide a clear delineation of subgroups at risk and thus, a combination of markers may be necessary. This seems difficult, especially because they are costly, invasive, or unsuitable for broad application. Nevertheless, there is an urgent need for biomarkers to diagnose AD early in its course, to differentiate it from other related diseases or subtypes. Furthermore, accurate biomarkers would be able to determine the clinical efficacy of novel neuroprotective strategies. With improved experimental design/sample preparation and implementation of advanced methodologies and analysis tools researches in biomarkers for AD will likely contribute significantly to managing this neurodegenerative disease in the years to come.

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Abbreviations: AD: Alzheimer's disease; APP: beta-amyloid precursor protein; CSF: cerebrospinal fluid; Gab2: GRB2-associated binding protein 2; ROS: reactive oxygen species; PKC: protein kinase C; HO-1: heme oxygenase-1

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