

ORIGINAL ARTICLE

ApoE Alleles and Tau Markers in Patients with Different Levels of Cognitive Impairment

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Background. The presence of brain hyperphosphorylated tau constitutes a hallmark of neurodegenerative disorders of the Alzheimer's type. This report describes the relationships between tau markers in the cerebrospinal fluid (CSF), the degree of cognitive impairment and the predictive value of genetic markers such the alleles of apolipoprotein E, namely, the presence of Apo- $\epsilon 4$, as part of a longitudinal study.

Methods. Three major groups of patients with ages ranging from 65–73 years were evaluated in this study ($n = 72$): Alzheimer's disease patients (AD), a group with mild cognitive impairment (MCI) and normal senile patients (NS). Hyperphosphorylated tau and tau dephosphorylated species at the Alzheimer-type epitopes in CSF samples were analyzed by ELISA assays using a battery of different monoclonal antibodies. ApoE was analyzed by PCR in blood samples.

Results. The levels of hyperphosphorylated tau were significantly higher in AD patients, but no statistical differences were found between the MCI and NS groups. However, the analysis of tau markers and cognitive impairment indicated the existence of two main subgroups within this population: MCI patients with a higher cognitive impairment as revealed by the total box score (TBS) >1.5 who exhibited phosphorylated tau patterns similar to the AD group, and patients with a mild impairment (TBS <1.5) with tau patterns similar to normal patients. In regard to ApoE, $\epsilon 4/\epsilon 4$ genotype was absent in the Chilean population analyzed, and only the $\epsilon 2/\epsilon 4$ genotype was significantly increased in both MCI and AD patients. A detailed analysis of the ApoE alleles, particularly $\epsilon 3$ and $\epsilon 4$, indicated a tendency to increase the $\epsilon 4$ allele in the MCI group with higher cognitive impairment and in AD patients.

Conclusions. Studies indicate that hyperphosphorylated tau is a good indicator of the degree of cognitive disorders in early stages of AD and that no clear correlation exists with the $\epsilon 4/\epsilon 4$ and $\epsilon 3/\epsilon 4$ genotypes, even though a higher proportion of $\epsilon 4$ allele in the MCI group with a more significant level of impairment and in AD patients was evidenced.

Key Words: Alzheimer's disease, Mild cognitive impairment, Cerebrospinal fluid, Tau protein, Apolipoprotein-E.

Introduction

Alzheimer's disease (AD) is the most common cause of dementia that has increased worldwide, even in countries with restrained economic resources, as life expectancy has

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markedly increased in the past two decades. AD is also a major public health problem and one of the most costly diseases in modern society (1,2). The main biological features of AD are a gradual neuronal dysfunction at early stages of the disease, alterations in brain vessels, followed by an increasing neuronal loss. These changes are accompanied by the formation in the brain of two main protein aggregates: the extraneuronal β -amyloid deposits or senile plaques (SP), and the presence of neurofibrillary tangles (NFTs) mainly composed by hyperphosphorylated tau protein (1,3).

These physiopathological features provide a framework for the analysis of biological markers that include amyloid peptide such as A β (1–42) with a higher capacity to self-aggregate, soluble oligomers of this peptide, and hyperphosphorylated forms of tau protein (4–7). There is clear evidence that CSF levels of this amyloid peptide are significantly decreased in AD patients, and that the hyperphosphorylated tau levels are increased (6–9). The patterns of posttranslationally modified tau in the CSF appear to be valuable markers for diagnosis at early stages of this disease (10). In spite of these findings, little is known about the changes of these markers according to cognitive impairments and how they are modified at early and late stages of this disease. Nevertheless, AD is a multifactorial disease, and several other factors contribute to the neurodegenerative process (11). Among them, oxidative stress promoting molecules and free radicals (12), including changes in the redox iron levels (13,14), proinflammatory cytokines (15,16) and neurotoxic agents appear to induce alterations in the normal signaling patterns of neurons and their communications with glial cells (17). Oxidative stress is a major pathological aspect of several neurodegenerative conditions and results from the generation of large amounts of reactive oxygen species (ROS), which can alter the structure of several molecules including proteins, lipids and nucleic acids, ultimately leading to cell degeneration and death (18,19).

Materials and Methods

Patients. We compared CSF samples from 72 elderly patients from the Hospital El Salvador in Santiago, Chile, out of 620 patients included in the longitudinal study, with different degrees of dementia. The age of the patients was between 65 and 73 years old, with a mean age of 69.4 ± 6.3 years. The educational level of the entire population analyzed had a mean of 12.1 ± 3.5 years of formal education. No significant differences were found among mean ages and mean educational levels of patients belonging to groups with normal cognitive status, mild cognitive impairment and Alzheimer's disease, even though a lower value for level of education in the subgroup of AD patients was observed. Controls were selected randomly among patients attending the Hospital service, following the appropriate informed consent protocols. Patients were from the

residential metropolitan area of Santiago, Chile that corresponds to about 40% of Amerindian population and 60% of Caucasian ancestors and who fulfilled the inclusion criteria of the study: 1) >60 years old; 2) free of significant underlying medical or neurological illness; and 3) willing to participate in the study, providing informed consent. Participants were recruited through the printed media representing all the social and ethnic groups in the country and underwent a multistage screening procedure. In the cases of demented subjects, informed consent for participation in the study was obtained also from their caregiver. The study and experimental protocols were previously approved by the Committee on Ethical Issues of the Faculty of Medicine, University of Chile. Clinical diagnosis of AD was made according to Alzheimer's criteria as outlined by the National Institute of Neurologic, Communicative Disorders and Stroke, and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) Work Group (20). The individuals were rated using a semi-structured interview and divided into three different groups for the study as already mentioned: subjects with a Normal Cognitive Status (NS, $n = 18$), Mild Cognitive Impairment (MCI, $n = 37$), and Alzheimer's Disease (AD, $n = 17$). Clinical evaluation of MCI was done according to Petersen's criteria (21). According to the neuropsychological battery of tests and quantification of the degree of cognitive impairment, two subgroups were detected in the MCI population: those with early cognitive impairment with total box score (TBS) between 0 (normal) and 1.5 and those with a higher cognitive impairment with TBS >1.5. The number of subjects for MCI with low cognitive impairment was 18, and subjects of MCI group with more advanced impairment were 19.

Application of semi-structured interview. Application of CDR ratings were obtained using a semi-structured interview specially adapted by Daly et al. (22) from the previously validated original version of Hughes et al. (23). This interview was specially adapted to be used with a population with mild impairments. It includes a standardized medical, neurological, and psychiatric examination, in addition to a semi-structured set of questions about functional status regarding activities of daily living (ADL) and instrumental daily living (IADL). The subjects, distributed across a range of cognitive function from no impairment to mild impairment, were evaluated by three independent interviewers. The questions that the three interviewers considered were not well understood by the subjects or collaterals were modified according to the language skills of our population. The ratings of the overall CDR scores were analyzed with a kappa index. A very high concordance was obtained.

These subjects underwent a comprehensive medical and neurological examination to ascertain that they were free from any significant pathology. Once the interview was completed and rated, the subjects in the study were administered a neuropsychological battery. CDR ratings were completed

with the interviewers blinded to the results of the neuropsychological test. The written interviews were scored by a reviewer who made his own rating judgment, blinded to the interviewer.

Neuropsychological battery of tests. The neuropsychological evaluation consisted of a Neuropsychological Battery of CERAD (24) that includes Folstein's MiniMental Test, Verbal Fluency, Boston Nomination Test (15 items), Learning Word List (10 items), and Praxis.

CSF samples. CSF samples were obtained by lumbar punctures performed early in the morning. CSF samples were stored in polypropylene tubes without preservative and frozen at the bedside on dry ice within minutes of withdrawal. Samples were kept at -80°C .

Measurement of CSF hyperphosphorylated tau pools. Cerebrospinal fluid samples were analyzed to obtain levels of hyperphosphorylated tau species by ELISA assays (10,25). Tau-5 monoclonal antibody was used for total tau, whereas AT8 and PHF1 antibodies were used for measurements of hyperphosphorylated tau and Tau-1 antibody to assess tau species unphosphorylated at the AD type epitopes. Tau-5 and Tau-1 monoclonal antibodies were generously donated by Dr. Lester Binder, and PHF1 was a donation from Dr. Peter Davis. AT8 is a commercial monoclonal antibody purchased from Immunogenetics, Belgium.

ApoE determinations. The ApoE genotypes were determined by PCR of DNA obtained from blood samples from the different patients (26). Essentially, DNA was extracted from lymphocytes, the ApoE gene was amplified by PCR, extracted and subjected to HhaI restriction enzyme, for further gel fractionation.

Statistical analysis. One-way ANOVA was used to test differences in mean values for the tau levels study in the CSF, and Bonferroni's post-hoc test was used for comparisons (GraphPad InStat, GraphPad Software, San Diego, CA). Differences were considered significant at the significance level of $p < 0.05$. χ^2 square analysis was performed for the analysis of $\epsilon 4$ alleles with respect to the different subsets of patients using $p < 0.05$.

Results

Levels of total and hyperphosphorylated tau species in the CSF. Analysis of total tau by ELISA showed no significant differences between the NS, MCI and AD groups, with values (mean \pm SD) of 0.42 ± 0.08 . Analysis of the levels of tau dephosphorylated at the Alzheimer's epitopes also showed no significant differences among groups of subjects

(Table 1). However, analysis of AT8 reactive hyperphosphorylated epitopes of Alzheimer's type showed significant differences between NS and AD (0.46 ± 0.09 and 0.60 ± 0.08 , respectively). Moreover, differences between MCI and AD (0.51 ± 0.09 and 0.60 ± 0.08 respectively) were also detected according with ANOVA tests.

When the MCI population ($n = 37$) was divided into two subgroups according to the level of cognitive impairment, significant differences were found between the MCI subgroup with TBS > 1.5 with higher cognitive impairment within the MCI group, and NS (0.54 ± 0.09 and 0.46 ± 0.09 , respectively), and no differences were found between the subgroup with TBS > 1.5 and the AD group (Table 2). No differences were also revealed when the MCI subgroup with TBS < 1.5 and NS were compared. However, a significant difference was evidenced when the MCI subgroup with TBS < 1.5 (0.48 ± 0.09) and the AD group (0.60 ± 0.08) were compared (Tables 1 and 2). Similar findings to those with AT8 were observed with the PHF1 monoclonal antibody that recognizes other hyperphosphorylated tau epitopes, characteristics of AD.

Relationships between the ApoE alleles, the levels of tau hyperphosphorylated epitopes and the degree of dementia. The distribution of ApoE genotypes was analyzed by PCR in blood samples from the different groups of patients. The genotype $\epsilon 2/\epsilon 3$ was found in slightly different proportion in all the NS, MCI and AD groups analyzed in the study, while $\epsilon 2/\epsilon 2$ was absent. The genotype $\epsilon 4/\epsilon 4$, found in AD subjects from populations in the northern hemisphere (27), was also absent in the 72 patients considered in this study. The correlation between the ApoE genotypes and the level of cognitive impairment in the different groups of subjects is shown in Table 3. In spite of the fact that homozygous genotype $\epsilon 3/\epsilon 3$ (~50%) and heterozygous genotype $\epsilon 3/\epsilon 4$ (~20%) were identified in similar proportions in all groups of subjects, genotype $\epsilon 2/\epsilon 4$ was absent in NS, present in 5.4% of MCI (10.5% in the MCI with TBS > 1.5), and increased to 17.6% in AD. On this basis, the presence of Apo $\epsilon 4$ allele was evaluated separately. The allele was identified in four cases of NS, ten cases of MCI and seven cases of AD. When $\epsilon 4$ allele was analyzed in the MCI subsets of patients, it was identified in three patients of the MCI population with early cognitive impairment (TBS < 1.5) and in

Table 1. CSF levels of hyperphosphorylated and desphosphorylated tau species

	NS ($n = 18$)	MCI ($n = 37$)	AD ($n = 17$)
Tau antibody	Mean \pm SD	Mean \pm SD	Mean \pm SD
AT 8	0.46 ± 0.09^b	0.51 ± 0.09^a	$0.60 \pm 0.08^{a,b}$
Tau-1	0.40 ± 0.05	0.39 ± 0.07	0.39 ± 0.07
PHF-1	0.38 ± 0.06^b	0.42 ± 0.06^a	0.59 ± 0.07

^aSignificant differences between MCI and AD (ANOVA).

^bSignificant differences between NS and AD.

Table 5. Demographic and cognitive performance data

	NS Mean ± SD	TBS <1.5 Mean ± SD	TBS ≥1.5 Mean ± SD	AD Mean ± SD
Total <i>n</i>	18	18	19	17
Age	67.5 ± 5.9 ^a	66.9 ± 4.6 ^a	71.8 ± 5.9 ^b	71.4 ± 6.6 ^b
Education	12.1 ± 3.2 ^a	13.6 ± 4.0 ^a	12.9 ± 3.2 ^a	9.6 ± 3.2 ^b
Sex	7M/11F	6M/12F	8M/11F	8M/9F
CERAD				
Trial 1	5.1 ± 1.50 ^a	4.67 ± 1.46 ^a	3.89 ± 1.56 ^a	1.94 ± 1.25 ^b
Trial 2	7.11 ± 0.98 ^a	6.67 ± 0.91 ^a	5.37 ± 1.57 ^b	3.06 ± 1.98 ^c
Trial 3	8.44 ± 0.78 ^a	8.22 ± 1.22 ^{a,b}	7.11 ± 1.52 ^b	3.82 ± 1.67 ^c
Learning	20.67 ± 2.10 ^a	19.56 ± 2.96 ^a	16.37 ± 3.61 ^b	8.76 ± 4.58 ^c
Delayed recall	7.44 ± 1.34 ^a	5.94 ± 1.66 ^a	4.21 ± 2.23 ^b	0.65 ± 1.32 ^c
Praxias	9.33 ± 1.64 ^a	9.11 ± 1.45 ^a	8.37 ± 1.67 ^a	5.24 ± 2.25 ^b

Note: Analyses reflect differences at 0.05 level for comparison of adjacent group. Similar letters (i.e., ^{a, b, c, d}) indicate no significant difference between pairwise comparisons.

TBS, total box score; NC, normal control; AD, Alzheimer's disease; M, male; F, female.

and a clear hyperphosphorylated tau pathology (11,12). This is sustained by *in vitro* studies on primary hippocampal cultures. At very early stages of the neurodegenerative process, tau dephosphorylations occurs on the typical AD epitopes due to changes in the ratios of kinases and protein phosphatases, namely, PP1 (12). At more advanced stages, the overactivation of cdk5 system is responsible for a marked increase in the phosphorylation of the AD epitopes, leading to a loss of tau function in controlling cytoskeletal dynamics (29). At longer times of the degenerative process, tau initiates self-aggregation to form straight filaments and later the paired helical filaments that conform the neurofibrillary tangles (33).

Considering that the allele $\epsilon 4$ of the apolipoprotein E system constitutes a major risk factor for AD (34), it was of interest to assess the relationships between the presence of this allele, the genotypes found in the three groups of patients and the extent of cognitive disorder. Because tau patterns exhibit dramatic variations in the CSF of these patients, it is also interesting to analyze the links between the nature of tau modifications and the presence of $\epsilon 4$ allele in the different subsets of patients evaluated. Even though the ApoE genotypes did not show major associations with the different groups of patients—NS, MCI and AD—the fine distribution of the alleles exhibited interesting differences. Thus, the tendency to increase the presence of $\epsilon 4$ allele in the whole MCI group and in AD patients was evidenced. The links of the allele $\epsilon 4$ with the subgroups of MCI patients with the higher cognitive impairment and those of AD group are based on inspection of Tables 3 and 4. Thus, an increase in the observed $\epsilon 4$ presence was evidenced for the MCI and AD groups, but statistical analysis indicated that differences were not significant probably because of the still small number of observations in the different groups of subjects. These findings are in agreement with the predicted results based on the observations on $\epsilon 4$ as a risk factor for AD but do not provide clear information to ascertain the association

of $\epsilon 4$ allele with the levels of cognitive impairment. In the context of this analysis, the finding of an absence of $\epsilon 4/\epsilon 4$ genotype in this group of Chilean subjects evaluated was of interest. Even though the $\epsilon 3/\epsilon 3$ genotype resulted to be similar for the three groups analyzed, the $\epsilon 2/\epsilon 4$ genotype was found in an increased proportion in the MCI group and significantly higher in AD group of patients.

Studies indicate that hyperphosphorylated tau is a good indicator of the degree of cognitive disorders in early steps of AD, and that no clear correlation exists with the $\epsilon 4/\epsilon 4$ and $\epsilon 3/\epsilon 4$ genotypes, even though a higher proportion of $\epsilon 4$ allele in the MCI group with a higher level of impairment and in AD patients was evidenced.

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