Polymorphism in the Cholesterol 24S-Hydroxylase Gene (CYP46A1) Associated with the APOE ϵ 3 Allele Increases the Risk of Alzheimer's Disease and of Mild Cognitive Impairment Progressing to Alzheimer's Disease

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Key Words

Alzheimer's disease · Mild cognitive impairment · CYP46A1 · APOE · 24S-Hydroxycholesterol · Cytochrome P450

Abstract

Background: Late-onset Alzheimer's disease (LOAD) is associated with changes in certain proteins, such as ApoE and Cyp46A1, of the elimination route for cerebral cholesterol. The main lipoprotein involved in its transport is ApoE whose ϵ 4 allele is the least efficient. However, the presence or absence of this allele does not determine the development of LOAD, which implies the existence of other susceptibility factors associated with the disease, such as the CYP46A1 gene that encodes the enzyme cholesterol 24S-hydroxylase. **Objective:** To find new data to contribute to the evaluation of whether the presence of the T allele in the polymorphic site rs754203 of the CYP46A1 gene leads to a greater risk of developing mild cognitive impairment (MCI) and LOAD. Further-

more, given the link between APOE and CYP46A1, we proceeded to relate both genotypes in each of the patient groups studied. **Methods:** We studied MCI and LOAD patients and also carried out an analysis of those MCI patients who progressed from a mild cognitive deterioration to a clinically evident Alzheimer's disease during the study. **Results:** The frequency of the CYP46A1-T allele in the LOAD patients with APOE ϵ 3 alleles is significantly higher with respect to the control group; the same occurs in the group made up of LOAD patients together with the MCI patients who progressed to LOAD. The risk of developing LOAD when this allelic combination exists is 2.262 times higher (95% CI 1.337–4.202). However, having the CYP46A1-T allele does not increase the risk of suffering from LOAD in carriers of the APOE ϵ 4 allele, probably because the transport of cholesterol is already affected in such patients and possibly masks the effect of the CYP46A1-T allele. **Conclusions:** The CYP46A1-T allele increases the risk of suffering from LOAD in persons carrying the APOE ϵ 3 allele.

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Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder that gives rise to a progressive cognitive deterioration and is characterized by the presence of neurofibrillary tangles and the deposition of amyloid- β protein (A β), a residue of the amyloid protein precursor [1, 2]. Furthermore, the establishment of late-onset Alzheimer's disease (LOAD) is associated with an increase in cerebral cholesterol. Cholesterol gives the membranes stability and fluidity; however, the excess, whether due to excessive synthesis or to liberation by cell death, needs to be removed from the brain.

The mechanism of transporting cholesterol in the organism is carried out through the lipoproteins. The binding of lipoproteins to the cells requires recognition by the cell receptors, for which ApoE possesses the highest affinity [3, 4]. ApoE is also the main transporter of cholesterol in the neuronal cell membrane [2, 5].

The gene that codifies ApoE has three alleles: ϵ 2, ϵ 3 and ϵ 4. Of these, the ϵ 4 allele is the least efficient and the one which confers a greater risk of suffering from LOAD [6–8].

However, not all the individuals with AD have at least one APOE ϵ 4 allele. This implies the existence of other genetic susceptibility factors associated with LOAD [9]. Among these might be the CYP46A1 (14q32.1) gene, which encodes the cholesterol 24S-hydroxylase enzyme belonging to the P450 cytochrome family, mainly expressed in the brain [10–12]. The cholesterol 24S-hydroxylase enzyme is involved in the major pathway for the elimination of brain cholesterol. Its function consists of adding a hydroxyl group to cholesterol, making it more permeable to the blood-brain barrier, thus allowing the maintenance of brain cholesterol homeostasis [13].

Previous studies have determined that an increase in the activity of Cyp46A1 produces an accumulation in the cerebrospinal fluid of 24S-hydroxycholesterol, which has been shown to be a potent neurotoxin in vitro. This compound could thus favour the development of the disease by promoting neuronal death [13–15]. On the other hand, the decrease in activity of Cyp46A1 favours the accumulation of cholesterol in the brain, which as a consequence leads to the formation of A β plaques [12]. Hence, the activity of the enzyme cholesterol 24S-hydroxylase seems to be closely related to the development of AD and its variants could be susceptibility factors. On these lines, Kölsch et al. [16] and Desai et al. [17] analysed the polymorphism rs754203 which involves a C/T transition, but they reported negative results in which no association was

observed between this polymorphism and LOAD. However, a later study by Papassotiropoulos et al. [18] found an association between the T allele and AD. Recently, Borroni et al. [19] and Combarros et al. [20] have determined once again an association between CYP46A1 and LOAD, but the susceptibility allele in this case is the rs754203-C of the cytochrome.

In view of the contradictory results previously reported, the purpose of this study consisted of the search for new data to contribute to evaluating whether the presence of the T allele in the rs754203 polymorphic site of the CYP46A1 gene confers a greater risk of developing LOAD. To this end, we studied patients with LOAD and also carried out an analysis of other patients who progressed during the study from having mild cognitive impairment (MCI) to clinically evident AD. In addition, having seen the link between APOE and CYP46A1, we proceeded to relate both these genotypes in each group of patients studied.

Materials and Methods

We carried out the study on a sample of Caucasian residents in the Basque Country. All individuals were selected after a neuropsychological examination (using NINCDS-ADRDA and DSM-IV criteria) to confirm the presence or absence of the disease, after obtaining informed consent. A series of 119 cognitively healthy controls were also analysed (clinical dementia ratio = 0). Control subjects and patients were recruited from several hospitals and old people's homes of the Basque Country [Txagorritxu and Cruces Hospitals and the following old people's homes: Aurora, Los Molinos, Olárizu, Los Arquillos and San Prudencio; both hospitals belong to the Basque Health Service (Osakidetza)].

The sample consists of 223 patients: 100 patients with LOAD, 100 patients with MCI and 23 patients who in the course of the study progressed from MCI to LOAD. We analysed 119 control subjects of comparable age and sex. Table 1 shows the mean age at onset and at collection of the patients, and the age at collection of the controls in all the studied cases. Table 2 shows the frequency for men and women in each population. Patients had been closely followed for the last 4 years through an annual visit. The 23 MCI patients progressed to LOAD in a variable period of time since the age at onset of MCI. The mean time of progression was 2.1 ± 1.4 years for the global population, 2.2 ± 1.8 years for men and 2.1 ± 1.3 for women.

Genetic Studies

The extraction of DNA was carried out using the proteolysis method and purification with phenol-chloroform on aliquots of 250 μ l of peripheral blood samples collected in EDTA anticoagulant and stored at -20°C . The DNA extracted was precipitated with ethanol and diluted to a final concentration of 30 ng/ μ l, on which genotyping was performed using the polymerase chain reaction (PCR) method and digestion with restriction enzymes.

APOE was amplified with the primers 112F and 158R, in the PCR conditions described by Wilton and Lim [21]. Digestion of the amplified product was carried out with *Hae*II and *Afl*III as described by Álvarez-Álvarez et al. [9].

One hundred and eighty-two base pairs containing the single nucleotide polymorphism rs754203 of the CYP46A1 gene were amplified with the primers described by Papassotiropoulos et al. [13] (forward: 5'-AATGCATGCTACCAAAAGAG-3' and reverse: 5'-AATCATTTGATTCCCAGGAC-3'). The PCR protocol used

corresponds to a touchdown with the following stages: 94°C, 5 min; 8 cycles at 94°C, 30 s, 59°C, 30 s, 72°C, 30 s; 27 cycles at 94°C, 30 s, 55°C, 30 s, 72°C, 30 s, and 72°C, 7 min, in a final volume of 25 µl. Ten microlitres of the PCR product were cut with 4 U of the restriction enzyme *Msp*I and another 10 µl with 2 U of *Hpa*II at 37°C overnight. The obtained fragments correspond to a 182-bp-long amplicon for the T allele and to two amplicons of 45 and 137 bp, respectively, for the C allele.

The fragments obtained were migrated on a 3% agarose gel stained with ethidium bromide and visualized under ultraviolet light.

Statistical Analysis

The allelic and genotypic frequencies were compared with the χ^2 and Fisher's exact test using the SPSS 12.0 statistical package. The criterion for significance was set at $p < 0.05$. The Guo and Thompson test [22] for Hardy-Weinberg equilibrium was performed with the Genepop 2.0 program. The odds ratios and the Kaplan-Meier analysis were calculated with SPSS 12.0 package. A multiple correspondence analysis [23–26] and a logistic regression [27] were also performed with the SPSS 12.0 package.

Results

The distribution of genotype and allele frequencies of APOE and CYP46A1 in LOAD, MCI and MCI to LOAD patients and controls is shown in table 3. As usual in the analysis of APOE in patients with LOAD and MCI, the frequency of the APOE ϵ 4 allele is much higher in the patients than in the controls, the reverse of what happens with the APOE ϵ 3 allele. The CYP46A1-T allele also shows higher frequencies in LOAD and MCI patients than in controls, although the difference is not so marked.

Table 1. Mean ages at onset and collection of patients and controls

	Total	Men	Women
Age at collection			
AD	75.09 $\pm$ 7.8	77.3 $\pm$ 6.8	75.4 $\pm$ 8.1
MCI	71.5 $\pm$ 8.7	72.2 $\pm$ 8.2	71.2 $\pm$ 8.9
MCI to AD	75.1 $\pm$ 7.9	70.7 $\pm$ 7.4	76.8 $\pm$ 7.6
Controls	77.6 $\pm$ 9.7	76.5 $\pm$ 8.7	78.7 $\pm$ 10.3
Age at onset			
AD	71.7 $\pm$ 8.3	73.1 $\pm$ 6.9	71.2 $\pm$ 8.7
MCI	68.6 $\pm$ 9.3	69.7 $\pm$ 8.2	68.1 $\pm$ 10.0
MCI to AD	73.1 $\pm$ 8.2	68.3 $\pm$ 8.0	78.9 $\pm$ 7.8

Table 2. Sex distribution frequencies in patients and controls

	AD	MCI	MCI to AD	Controls
Men	0.24	0.36	0.27	0.38
Women	0.76	0.63	0.73	0.62

Table 3. Genotype and allele frequencies of the APOE and CYP46A1 genes in patients and controls

APOE	n	Genotypes					Alleles		
		2/3	2/4	3/3	3/4	4/4	2	3	4
Controls	119	0.06	0.00	0.78	0.16	0.00	0.03	0.89	0.08
AD	100	0.04	0.02	0.40	0.41	0.13	0.03	0.62	0.35
MCI	100	0.06	0.01	0.54	0.30	0.09	0.04	0.72	0.24
MCI to AD	23	0.00	0.00	0.61	0.39	0.00	0.00	0.80	0.20
CYP46A1	n	Genotypes			Alleles				
		TT	TC	CC	T	C			
Controls	119	0.56	0.39	0.05	0.75	0.25			
AD	100	0.65	0.31	0.04	0.81	0.19			
MCI	100	0.63	0.33	0.04	0.80	0.20			
MCI to AD	23	0.65	0.31	0.04	0.75	0.25			

Table 4. Analysis of Pearson's χ^2 as a function of the APOE and CYP46A1 alleles

	CYP46A1		
	T allele	C allele	p (χ^2) ¹
APOEε2			
AD	6 (1)	0	NS (1.000)
AD + MCI to AD	6 (1)	0	NS (1.000)
MCI	6 (0.86)	1 (0.14)	NS (1.000)
Control	6 (0.86)	1 (0.14)	
APOEε3			
AD	110 (0.88)	15 (0.12)	0.009
AD + MCI to AD	143 (0.88)	19 (0.12)	0.003
MCI	119 (0.83)	25 (0.17)	NS (0.186)
Control	162 (0.76)	50 (0.24)	
APOEε4			
AD	45 (0.65)	24 (0.35)	NS (0.597)
AD + MCI to AD	49 (0.63)	29 (0.37)	NS (0.794)
MCI	34 (0.69)	15 (0.31)	NS (0.402)
Control	11 (0.58)	8 (0.42)	

¹ Versus control.

Furthermore, we carried out an analysis of the allele associations of CYP46A1 and APOE. Table 4 shows the results achieved. As can be seen, the frequency of the allele CYP46A1-T in the APOE ϵ 3 LOAD patients is raised compared to the controls. This difference is statistically significant ($p = 0.009$) and was also observed when we analysed the group made up of LOAD patients together with the MCI patients who developed LOAD during the course of the study ($p = 0.003$). The risk of developing LOAD when this allele combination occurs is 2.262 times higher (95% CI 1.337–4.202). The group of MCI patients with the APOE ϵ 3 allele also shows a higher frequency of CYP46A1-T than the controls, but the difference does not reach statistical significance. The differences in the distribution of the CYP46A1 alleles were not statistically significant for patients with APOE ϵ 2 and APOE ϵ 4 alleles (table 4).

The separate study of the group of MCI patients who developed LOAD during the period under study did not show statistically significant differences in the CYP46A1 and APOE allele association with respect to the controls. The results obtained after carrying out Kaplan-Meier's determination, which assesses the progress of the disease over time, did not show significant differences either (data not shown).

A multiple correspondence analysis was carried out. This analysis included the variables 'patient clinical group', 'patient/control health quality', 'APOE and CYP46A1 genotypes', 'gender' and 'academic level'. As a result, an evident association between the APOE ϵ 4 allele and the academic level was obtained. Moreover, this association grows stronger when the academic level is compared to the homozygotic genotype of the APOE ϵ 4 allele. In addition, the highest academic level was found to be associated with the health quality of the patients/controls. Nonetheless, when the academic level stood for a predicting variable of the disease in a logistic regression, it did not show a significant association with the patient/control health quality (data not shown).

Discussion

In the majority of the studies carried out on LOAD, the presence of the APOE ϵ 4 allele has been associated with a higher risk of suffering from AD; however, the presence of this allele does not necessarily lead to the development of the disease, as there are many patients with LOAD who do not have the APOE ϵ 4 allele [9, 28]. In our study, we too have observed that there is also a high incidence of the APOE ϵ 3 allele in individuals with LOAD (table 1), hence other genic factors associated with LOAD must exist. Papassotiropoulos et al. [18] demonstrated a raised frequency of the T allele of the CYP46A1 gene in the group of patients with AD compared to controls. These results seem to indicate that this polymorphism in the CYP46A1 gene is a new risk factor that deserves to be studied in other groups of patients.

In the present piece of work, we have studied the allele distribution of this polymorphism in LOAD patients and in a group of controls. We found a higher frequency of the CYP46A1-T allele than of the CYP46A1-C allele both in patients and in controls, which matches with the results obtained in several other studies [16–20]. Apart from the LOAD patients, we also studied a group of patients with MCI whose allele frequency distribution of the CYP46A1 proved to be similar to that found in the LOAD and control groups.

The studies on the association between APOE and CYP46A1 carried out to date have produced contradictory results. On the one hand, Kölsch et al. [16] and Desai et al. [17] did not conclude that there was any type of association between the two markers and the risk of suffering from AD; these results were corroborated in

recent studies [29–32]. On the other hand, Papassotiropoulos et al. [18] described a synergy between the alleles APOE ϵ 4 and CYP46A1-T that was found again in a study carried out by Wang et al. [33] in the Chinese population. Likewise, Borroni et al. [19] described this same synergy but between the alleles APOE ϵ 4 and CYP46A1-C. In both cases, the risk of suffering from LOAD was increased. Finally, Combarros et al. [20] have detected a predisposition to LOAD associated with the genotype CYP46A1 C/C, independent of the genotype of APOE.

In our study, we have found an association between the CYP46A1-T allele and the APOE ϵ 3 allele; we did not observe the significant increase in the CYP46A1-T allele in patients with APOE ϵ 4 reported by Papassotiropoulos et al. [18]. In the same way, the analysis of 23 patients initially diagnosed as having MCI that developed into AD during the study revealed an increase in the frequency of the CYP46A1-T allele associated with APOE ϵ 3. These results differ from those obtained by Papassotiropoulos et al. [18] in that they do not show a synergy between the alleles CYP46A1-T and APOE ϵ 4. One possible cause for the controversy found between the results obtained by Papassotiropoulos et al. [18] and the ones obtained in this study may be an erroneous genotyping. This explanation has been discarded for two reasons: (a) the studied samples are in Hardy-Weinberg equilibrium and (b) the genotype has been verified using a second restriction enzyme. As the restriction enzyme *MspI* used to differentiate between the T or C alleles recognizes the C allele sequence in the PCR product, partial digestion or the lack of digestion may be erroneously interpreted as genotype TT, thus increasing the frequency of the T alleles in the sample analysed. In order to make sure that the genotypes obtained here were correct, we re-analysed our samples using the isoschizomeric enzyme *HpaII*. Although this enzyme is sensitive to the state of methylation of the DNA, so that it cleaves only when the CpG bases of the target are not methylated, this characteristic has no value in the case of digestion of the PCR products; for this reason, the use of this enzyme is helpful to verify the genotypes determined with the *MspI* enzyme. In all cases, we found good concordance; thus, it is reasonable to think that the discrepancy detected between this study and the study by Papassotiropoulos et al. [18] is not due to genotyping errors.

Various different hypotheses have been established on the influence of the activity of this cytochrome on the appearance of LOAD. In the first stages of AD, an increase in the ratio of 24S-hydroxycholesterol/cholesterol

has been observed, while in the later phases, this ratio declines [16]. The reason for this is associated with an increase in the activity of the cytochrome, produced in the astroglia, in the first phases of the disease to compensate for the liberation of cholesterol originating in the degradation of the neuronal membrane [2, 16]. Later on, as the disease progresses, liberation of cholesterol is so much raised that the cytochrome can no longer compensate for the increase, to which is added the decline in the synthesis of Cyp46A1 in the neurone because of its degeneration. Thus, the lack of solubility of the cholesterol impedes its passage through the blood-brain barrier, it accumulates in the brain and contributes to the formation of the A β plaques and the advance of AD [13]. From a molecular perspective, Borroni et al. [19] obtained evidence of an association between the allele CYP46A1-C and LOAD, while Papassotiropoulos et al. [18] and our present study associate the CYP46A1-T allele with the disease. In the face of this situation, it is necessary to reflect on the difference between these results; it should be taken into account that the rs754203 polymorphism is located in an intronic region, upstream from the 3 exons of the CYP46A1 gene, and thus it is not clear how it acts on the 24S-hydroxycholesterol/cholesterol ratio. This polymorphism does not affect the sites of splicing as it does not correspond either to the 5'-donor or to the 3'-acceptor splice recognition motif of exons 3 and 4. Therefore, the branch site sequences have been shown to be located in an area 6–59 bp downstream of the acceptor splice site [16]. Thus, the difference between alleles associated with AD in the various studies may be due to a linkage with some other polymorphism situated in the coding region or sites of splicing capable of reducing the functionality of the CYP46A1 gene, so that in the group of individuals studied by Borroni et al. [19] this polymorphism may be associated with the C allele of the rs754203, while in the population studied by Papassotiropoulos et al. [18] and in the present study the association takes place preferably with the T allele.

On the other hand, it should also be taken into consideration that Papassotiropoulos et al. [18] determined a synergism between the CYP46A1-T allele and the APOE ϵ 4 allele in the development of LOAD, while in our study we observed an association between the CYP46A1-T allele and the APOE ϵ 3 allele. In addition, in the literature, the APOE ϵ 4 allele is considered a risk factor, hence the fact that in our study we only obtained a significant difference with the APOE ϵ 3 allele may mean that in the APOE ϵ 4 individuals the susceptibility associated with

this allele is sufficient to allow the development of the disease, masking the effect associated with LOAD of other genes, while, on the contrary, in individuals with APOE ϵ 3, the effect on the metabolism of cholesterol is visible.

In conclusion, our results determine that the CYP46A1-T allele increases the risk of suffering from LOAD in individuals in whom the APOE ϵ 3 allele is present. However, the different origins of the population groups selected should be taken into consideration, as these could directly affect the results obtained. The fact that these results might be confirmed running a check on the post-mortem definite AD samples has also been taken into account since the individuals studied here were diagnosed as probable AD. For this reason, addi-

tional studies are required to confirm the role played by 24S-hydroxycholesterol in the development of AD and the influence of the polymorphisms of the CYP46A1 gene which codifies the 24S-cholesterol hydroxylase enzyme.

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