SHORT REPORT

APOE promoter polymorphisms do not confer independent risk for Alzheimer's disease in a French population

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The apolipoprotein E (*APOE*, gene; apoE, protein) isoforms are associated with differential risk of Alzheimer's disease (AD). An additional involvement of *APOE* promoter polymorphisms in AD risk has recently been suggested by several studies. Indeed, three polymorphisms of the *APOE* regulatory region (–219 G/T, –427 C/T and –491 A/T) have been found associated with AD even after adjustment on the apoE status. We analysed these three promoter region polymorphisms in a large French case-control study (388 AD cases and 386 controls). We found that the –427 T and –491 A alleles were associated with an increased risk of developing AD, but not the –219 G/T alleles. However, a strong linkage disequilibrium was observed between the alleles of these promoter region polymorphisms and the *APOE* coding region alleles. We therefore retested association after adjustment on apoE status and found that the sole association which remained significant was the association with the –427 T allele. The α level was equal to 0.03 (0.09 after Bonferroni correction for multiple comparisons). Analysis of promoter haplotypes also yielded non-significant results. Thus our study does not reinforce the hypothesis of an independent involvement of the *APOE* promoter region polymorphisms in AD risk. *European Journal of Human Genetics* (2000) 8, 713–716.

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Since the initial report of an association between late-onset Alzheimer's disease (AD) and the apolipoprotein E (*APOE*, gene; apoE, protein) ϵ 4 allele,¹ more than 100 association studies have established the involvement of *APOE* in AD. Carrying an ϵ 4 allele increases the risk of AD in an allele dose manner: a meta-analysis found that the odds ratio (OR) is 3.2 (95% CI [2.8–3.8]) for individuals with one ϵ 4 allele and 14.9 (95% CI [10.8–20.6]) for those homozygous for the ϵ 4 allele.² The impact of the ϵ 4 allele is present in early as well as in late-onset AD, but is highest between ages 60 and 80.^{2,3}

The presence of an *APOE* ϵ 4 allele is neither sufficient nor essential for the development of AD. Other factors (genetic and/or environmental) should interact with the *APOE* geno-

type to determine the risk of AD. Recently it has been suggested that polymorphisms located in the regulatory region of the *APOE* gene could confer a risk of AD, even after adjustment on the apoE status. Three polymorphisms located respectively at –219 bp (Th1/E47cs), –427 bp and –491 bp of the transcriptional start site have been described. These biallelic polymorphisms are: –219 G/T,^{4,5} –427 C/T^{5,6} and 491 A/T^{5–14} and the at-risk alleles for AD are –219 T, –427 C and –491 A. For two promoter polymorphisms (–427 and –491), allelic variants have been coupled with a reporter gene and transfected in astrocytic cells showing that haplotypes associated with the risk of AD are also associated with a higher transcription activity.⁶ Therefore, an increase in expression of *APOE* could be the physiological mechanism responsible for the allelic association between the promoter region polymorphisms and AD. This conclusion is supported by the demonstration that subjects homozygous for the at-risk allele at the –491 locus (–491 AA) have increased

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plasma apoE levels.⁸ In contrast, the biological effect of the -219 alleles is controversial: Artiga *et al*¹⁵ have shown that, in hepatoma cells, the T to G substitution provoked an increase in promoter activity whereas Lambert *et al*⁶ have reported some evidence that the T allele may increase the relative expression of the *APOE* ϵ 4 allele in AD. A close examination of the data shows that for each polymorphism, the allelic association is inconstant among studies. The present study was undertaken to evaluate the role of the *APOE* promoter region, conditional on the presence of different apoE isoforms, in a large French population of 388 AD cases and 386 controls.

Materials and methods

Populations

All subjects enrolled in this study were Caucasian living in France. Unrelated AD patients were ascertained over a 5-year period through consecutive admissions in several university hospitals in France. The diagnosis of probable AD was established according to the criteria of NINCDS-ADRDA.¹⁶ Age at onset was defined as the age at which the patient or his family first noticed the symptoms required for the diagnosis. Patients belonging to families characterised by autosomal dominant inheritance of an AD gene with complete penetrance by age 60 were excluded from the analysis. In all, 388 AD patients were finally included (40.7% men; age at onset = 65.4 ± 9.4 years, range [40–90]).

Patients were compared with an age and sex-matched control sample. All controls were screened for cognitive functions. None of them showed symptoms of dementia. A total of 386 control subjects was included (45.6% men; age at examination = 66.9 ± 10.4 years, range [43–92]). Informed consent was obtained for each subject either directly or from a legal representative. The age and gender distributions did not differ significantly between AD patients and controls ($P = 0.06$ and $P = 0.17$, respectively).

Genetic analysis

DNA was extracted from blood lymphocytes using standard methods. *APOE* genotypes were determined by restriction enzyme digestion.¹⁷ The -491 and -427 genotypes were determined by nested PCR and double digestion by *DraI* and *AluI* restriction enzymes, as described.⁶ The -219 genotype was determined by PCR using a forward mismatched primer as described by Lambert *et al*.⁴ To enhance the specificity of the PCR reaction the reverse primer was redesigned as follows: 5'AGGATCCCAGACTTGTCCAATTATA3'. PCR reactions were performed in a total volume of 50 μ l containing 1.30 U Taq DNA polymerase, 2 mM $MgCl_2$, 200 μ M dNTP and 0.2 μ M of each primer. The PCR consisted of 40 cycles of 30 s at 94°C, 30 s at 58°C and 30 s at 72°C, preceded by 2 min at 94°C and followed by 3 min at 72°C. The 231 bp PCR product was purified using the GeneClean III kit (BIO 101, Vista, CA, USA) and then digested with 2.5 U of *MvaI* restriction enzyme

at 37°C overnight. Restriction fragments were separated on a 4% agarose gel and visualised using Gelstar (FMC Bio-Products, Rockland, Maine, USA). The T allele yielded a 49 bp fragment, whereas the G allele yielded two fragments of 31 + 18 bp. Three constant bands of 69 bp, 60 bp and 53 bp were also observed.

Statistical analysis

SAS software release 6.12 was used (SAS Institute, Cary, NC, USA). Univariate analysis was performed using a χ^2 test or Fisher exact test when necessary. In the multivariate analysis, we coded the genotypes of each subject as dummy variables according to the tested hypotheses: carrier of at least one ϵ 4 allele vs non-carrier of ϵ 4 allele; non-carrier of the -219 G allele vs carrier of at least one -219 G allele: TT vs GG + GT; non-carrier of the -427 C allele vs carrier of at least one -427 C allele: TT versus CC + CT; non-carrier of the -491 T allele vs carrier of at least one -491 T allele: AA vs TT + AT. The effects of these variables on the disease were assessed with a multiple logistic regression model adjusted for age and gender. Odds ratios (OR) are presented with 95% confidence intervals (95% CI).

Haplotypes were estimated by the EH program.¹⁸ This program allows testing for the existence of linkage disequilibrium between the alleles of two or more loci and estimation of haplotype frequencies.

Results

No deviations from the Hardy-Weinberg equilibrium were observed among controls for any of the polymorphisms. As expected, the presence of at least one ϵ 4 allele was associated with an increased risk of AD (OR = 4.02 [95% CI, 2.97–5.44]) (data not shown). We first studied the association of each promoter polymorphism with AD (Table 1). Compared with controls, AD patients had higher allele frequencies for -219 T, -427 T and -491 A, which led to an excess of homozygotes -219 TT, -427 TT and -491 AA. Differences in the genotype and allele distributions were significant only for the -427 and -491 polymorphisms. The OR for developing AD in subjects homozygous for the -427 T allele was 1.58 (95% CI, 1.08–2.31), and the OR for developing AD in subjects homozygous for the -491 A allele was 1.52 (95% CI, 1.10–2.09), after adjustment for age and gender (Table 2).

We next investigated the existence of linkage disequilibrium between these alleles, and between them and the ϵ 2, ϵ 3 and ϵ 4 alleles. No linkage disequilibrium was found between the promoter region polymorphisms alleles themselves (data not shown). In contrast, there was significant evidence for a strong linkage disequilibrium between *APOE* coding region polymorphisms and each of the three promoter polymorphisms. The -219 T, -427 T and -491 A alleles were statistically more frequently associated with the ϵ 4 allele than with the other *APOE* alleles ($P = 6.10^{-9}$, $P = 4.10^{-6}$, $P = 6.10^{-10}$, respectively). To eliminate possible confounding effects due

Table 1 Distribution of the -219, -427, -491 alleles and of the corresponding genotypes in controls and AD patients

	Allele		Genotype		
-219	G	T	GG	GT	TT
Controls	399 (0.52)	373 (0.48)	101 (0.26)	197 (0.51)	88 (0.23)
AD	373 (0.48)	403 (0.52)	89 (0.23)	195 (0.50)	104 (0.27)
-427	C	T	CC	CT	TT
Controls	84 (0.11)	688 (0.89)	5 (0.01)	74 (0.19)	307 (0.80)
AD	55 (0.07)	721 (0.93)	1 (<0.01)	53 (0.14)	334 (0.86)
-491	A	T	AA	AT	TT
Controls	635 (0.82)	137 (0.18)	258 (0.67)	119 (0.31)	9 (0.02)
AD	676 (0.87)	100 (0.13)	295 (0.76)	86 (0.22)	7 (0.02)

Frequencies are indicated in brackets.

Table 2 Adjusted OR estimated by multivariate logistic regression

OR [95% CI]	-219 TT vs GG + GT	-427 TT vs CC + CT	-491 AA vs TT + AT
Adjusted for age and gender	1.23 [0.88–1.70]	1.58* [1.08–2.31]	1.52* [1.10–2.09]
Adjusted for age, gender and including the three promoter polymorphisms and apoE status	0.96 [0.68–1.39]	1.56* [1.04–2.34]	1.22 [0.87–1.71]

* $P < 0.05$; 95% CI is indicated in brackets.**Table 3** Distribution of haplotypes H1, H2 and H3 in controls and AD cases (1), conditionally to the APOE $\epsilon 4$ (2) and $\epsilon 3$ (3) alleles

	Haplotypes	Controls	AD cases	P
(1)	H1	293 (0.379)	302 (0.389)	0.0008
	H2	273 (0.354)	326 (0.420)	
	H3	206 (0.267)	148 (0.191)	
(2)	$\epsilon 4$ – H1	34 (0.306)	80 (0.279)	0.43
	$\epsilon 4$ – H2	70 (0.631)	178 (0.620)	
	$\epsilon 4$ – H3	7 (0.063)	29 (0.101)	
(3)	$\epsilon 3$ – H1	258 (0.432)	227 (0.487)	0.13
	$\epsilon 3$ – H2	188 (0.315)	142 (0.305)	
	$\epsilon 3$ – H3	151 (0.253)	97 (0.208)	

Frequencies are indicated in brackets; distribution of haplotypes differs significantly between controls and AD cases (χ^2 , 2 df); distributions of haplotypes between controls and AD cases conditionally to the APOE $\epsilon 4$ or APOE $\epsilon 3$ allele are no more significantly different.

to this linkage disequilibrium, we performed a multivariate logistic regression including the three promoter polymorphisms and the APOE genotype in the same model, in addition to age and gender. After adjustment, the sole association which remained significant was that with the -427 T polymorphism (Table 2). When a Bonferroni correction for multiple comparisons was performed, this association of the -427 T allele with AD did not reach the significance level. The corrected significance level was 0.09 and the CI of the OR was [0.95–2.56] for a corrected α level equal to 0.05/3 (three test were realised at the final stage of the logistic regression analysis).

We then considered the promoter polymorphisms as a single haplotype including three DNA markers at position

-219, -427 and -491. Since only two haplotypes were frequent in controls, we decided to merge the other six into one single class. The three promoter polymorphisms were thus considered as a marker H of the APOE promoter with three haplotypes. The most frequent haplotype (-219 G--427 T--491 A) was named H1, the second (-219 T--427 T--491 A) H2 and the last H3. The distribution of these three haplotypes differed significantly between cases and controls ($P = 0.0008$) (Table 3). The haplotypes H1 and H2 were associated with an increased risk of AD when H3 was taken as a reference (OR[H1 vs H3] = 1.44, 95% CI [1.10–1.87]; OR[H2 vs H3] = 1.66, 95% CI [1.28–2.17]). When the haplotypes distribution was stratified according to the APOE alleles, the difference between cases and controls was

no more statistically significant, either between the $\epsilon 4$ allele AD cases and controls ($P = 0.43$), or between the $\epsilon 3$ AD cases and controls ($P = 0.13$) (Table 3).

Discussion

In our study, one of the three promoter region polymorphisms (-219G/T) showed no allelic association with AD even after adjustment on age, gender and apoE status. This result is consistent with those of Artiga *et al.*,⁶ who had found no significant association between the -219 polymorphism and risk of AD, but not with those of Lambert *et al.*^{4,5} Concerning this polymorphism, we have calculated that our sample had near 98% power to detect an effect of size equivalent to that reported by Lambert *et al.*⁴ The lack of association in our sample compared with that of Lambert *et al.* is explained both by an increase of the G allele in patients and by a decrease in controls in the present study. Nevertheless, we confirm the strong linkage disequilibrium, found by Lambert *et al.*⁴ between the -219 alleles and the *APOE* coding region alleles.

Our results concerning the -491 polymorphism perfectly match those obtained by Lambert *et al.* in a French population: the risk associated with the -491A allele is not independent of the apoE status.^{5,13} The only factor conferring an additional risk of AD, even after adjustment for the apoE status, is the -427T allele. Lambert *et al.* also observed a trend toward an excess of the T allele in their French sample, although this trend did not reach statistical significance.⁵ This result is unexpected since the allele conferring a higher transcriptional activity is the C allele, which was associated with AD in the Spanish population.⁶ Thus, the allelic association with the -427T allele is difficult to reconcile with the biological role assigned to this allele and we prefer the alternative explanation that the -427T allele is not functionally related to the additional risk of AD but is instead in linkage disequilibrium with a putative causative factor. Finally, no result remained statistically significant after the Bonferroni correction, the drastic effects of which are well known. It should be emphasised that no attempt to correct for multiple testing was made in other studies.^{4,6,7} In order to circumvent the problem posed by correction for multiple testing (which could lead to false negative results), we then analysed the three promoter polymorphisms as a single haplotyped marker with three alleles. Thus the number of tests was reduced and the study had more power to detect a possible role of the *APOE* promoter. However this approach also yielded negative results. We conclude that our results do not support the existence of an independent risk of AD conferred by *APOE* promoter polymorphisms.

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