Risk for Alzheimer's disease correlates with transcriptional activity of the *APOE* gene

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While the ε4 allele of apolipoprotein E (*APOE*, gene; ApoE, protein) is widely accepted as a major genetic risk factor for the late onset form of Alzheimer's disease (AD), recent evidence points to variations in ApoE levels as another important factor. We have previously reported that a common variant in the regulatory region of *APOE* (–491A) is associated with risk for late onset AD. In this report we analyze the association of another *APOE* promoter polymorphism (–427T/C) with AD in two case–control clinical samples and demonstrate a correlation between *APOE* promoter transcriptional activity and risk for AD. The association studies show that the allelic variant (–427C) and the haplotype [–491A–427C] of the *APOE* promoter are associated with increased risk for AD. Study of the transcriptional activity of the common haplotypes defined by combination of the –491 and –427 alleles indicated that the risk for late onset AD positively correlates with transcriptional activity of the *APOE* gene, suggesting that increases in the local expression of ApoE could be responsible for the association of *APOE* promoter polymorphism with AD.

INTRODUCTION

Alzheimer's disease (AD), the most common cause of dementia in the elderly, exists in both familial and sporadic forms. Genetic studies have led to the identification of three genes, β-amyloid precursor protein (APP) (1), presenilin-1 (2) and presenilin-2 (3), which, when mutated, can cause autosomal dominant forms of familial AD. However, mutations in these three genes are responsible for only a small fraction of AD cases, while the vast majority of patients with AD presents with a late age at onset and shows non-Mendelian inheritance. The absence of Mendelian inheritance indicates that late onset AD is a complex disease in which genetic and non-genetic factors are likely to be interacting. The ε4 allele of apolipoprotein E (*APOE*, gene; ApoE, protein) has been associated with an increased risk of developing AD (4). However, many AD cases have no *APOE* ε4 alleles. Thus it is likely that there are additional AD risk factors, both genetic and environmental, still to be identified.

We have screened for genetic variability in the proximal promoter region of *APOE* (nt –1017 to +406) and observed three new polymorphic sites (–491, –427 and –219) (5) and a previously described one (+113) (6) within this region. We have recently reported that one of these variants (–491A) is associated with risk for AD (7). We now report that the allelic variant –427C of the *APOE* promoter is also associated with AD, whereas we found no significant association of –219 and +113 polymorphisms with risk for AD. Results from others have suggested that the –219 polymorphism acts as a modifier of ApoE4-associated risk (8). Study of the transcriptional activity of the haplotypes defined by combination of the –491 and –427 alleles indicates that the risk for late onset AD correlates with transcriptional activity of the *APOE* gene.

RESULTS

During our screening of the transcriptional regulatory region of the *APOE* gene a novel polymorphic site (–427T/C) was identified (5). The possible genetic association of this polymorphism with risk for AD was studied in a Spanish case–control sample, in which we had previously observed an association between the polymorphism at position –491 of the *APOE* promoter and risk for AD (7).

The genotype and allele distributions for the –427T/C polymorphism are shown in Table 1. The TC genotype and the C allele were more frequent, although differences did not achieved statistical significance, in the AD group than in the controls, suggesting that they were associated with an increased risk for AD [odds ratio (OR) 1.87, 95% confidence interval (CI) 0.90–3.91 for the TC genotype and OR 1.78, 95% CI 0.88–3.62 for the C allele].

Table 1. *APOE* 427T/C genotype and allele frequencies (f) in Spain; age at onset >60 years

ApoE4 status	Genotype: *n* (f)			Allele: *n* (f)	
	TT	TC	CC	T	C
Whole sample					
Cases	90 (0.82)	20 (0.18)	0	200 (0.91)	20 (0.09)
Controls	118 (0.89)	14 (0.11)	0	250 (0.95)	14 (0.05)
ApoE4 non-carriers (–/–)					
Cases	28 (0.72)	11 (0.28)	0	67 (0.86)	11 (0.14)
Controls	103 (0.90)	12 (0.10)	0	218 (0.95)	12 (0.05)
Carriers (–/+, +/+)					
Cases	62 (0.87)	9 (0.13)	0	133 (0.94)	9 (0.06)
Controls	15 (0.88)	2 (0.12)	0	32 (0.94)	2 (0.06)

$2 \times 2\ \chi^2$ tests to compare TT and TC genotype frequency in AD versus controls were performed: whole sample, $P > 0.05$; ApoE4 carriers, $P > 0.05$; ApoE4 non-carriers, $P = 0.007$.

In order to determine whether the variation in risk for AD associated with the –427T/C polymorphism was independent of the risk conferred by ApoE4, the sample was stratified on the basis of the absence or presence of the ApoE4 allele (Table 1). No difference in the frequency of the –427C allele or the –427TC genotype was observed between the AD patients and controls bearing one or two ApoE4 alleles, but the –427C allele was clearly associated with increased risk for AD in individuals bearing no ApoE4 alleles (OR 3.00, 95% CI 1.26–7.07). Similarly, the –427TC genotype was associated with a >3-fold increase in risk for AD in the same subpopulation (OR 3.37, 95% CI 1.35–8.45), indicating that the variation in risk for AD conferred by the –427T/C polymorphism is only apparent in individuals not carrying any ApoE4 allele. The same trend was observed in the US group of individuals bearing no ApoE4 alleles (OR 1.40, 95% CI 0.65–3.01 for the TC genotype), although the differences in allelic frequencies did not achieve statistical significance in this population.

Since the –427T/C and –491A/T polymorphisms are located very close to each other within the proximal 5′-flanking region of the *APOE* gene and both of them appeared to be associated with risk for developing AD, we analyzed their effect on AD risk when combined into haplotypes. We first analyzed the [–491–427] haplotype distribution in both the Spanish and US samples. A combined digestion of an *APOE* fragment spanning nt –512 to –285 with *Dra*I and *Alu*I was performed, which allowed identification of the four haplotypes and the 10 genotypes defined by the combination of the –491 and –427 alleles. Haplotype [A-T] [–491–427] was the most common, followed by [T-T] and [A-C]. No subjects with the T-C haplotype were found in either sample. The distribution of these haplotypes in genotypes was close to that expected under Hardy–Weinberg equilibrium (χ^2 test, $P > 0.5$). Furthermore, no statistically significant evidence of linkage disequilibrium between these polymorphisms was detected (χ^2 test, $P = 0.3$). The most frequent genotype was [A-T]/[A-T], followed by [A-T]/[T-T] and [A-T]/[A-C]; these three genotypes accounted for >92% of both the Spanish and St Louis control populations. The frequency of genotypes [T-T]/[T-T] and [A-C]/[T-T] was <5% and the frequencies of the other genotypes ranged from 0 to 2%. The distribution of the major haplotypes and genotypes in the Spanish and St Louis samples is shown in Table 2.

Since our previous study had revealed differences in transcription associated with the –491 polymorphism (5,7), we then examined the functional characteristics of the major [–491–427] haplotypes present in our populations. The *APOE* proximal promoter activity of the three haplotypes ([T-T], [A-T] and [A-C]) was measured in U87 human astrocytoma cells using the same luciferase-based transient transfection system employed in our previous studies (5,7,19). As shown in Figure 1, transcriptional activity of haplotypes [A-T] and [A-C] were increased by 30 and 100%, respectively, over that of haplotype [T-T], suggesting that the presence of the alleles conferring increased risk for AD (–491A and –427C) is associated with increased constitutive levels of *APOE* promoter activity in human astrocytoma cells. The three haplotypes [T-T], [A-T] and [A-C] were thus named minus (–), even (*x*) and plus (+), respectively, according to their transcriptional activity. Additional information was obtained using oligonucleotide probes corresponding to the –491 and –427 allelic forms in electrophoretic mobility shift assays, with freshly prepared nuclear extracts from U87 cells. As shown in Figure 2, one of the three specific band shifts detected by the 427T probe (arrows) was not detected by the 427C probe, suggesting that the T→C substitution hinders binding of the probe to a nuclear protein. This finding is in agreement with the data obtained in our previous work using the 491 probes (7) and strongly suggests that differential transcriptional activities of the three [–491–427] haplotypes encountered in astrocytoma cells are a consequence of differential binding to nuclear proteins present in these cells.

Table 2. *APOE* [–491–427] major genotype and haplotype frequencies (f); age at onset >60 years

	Genotype: *n* (f)			Haplotype: *n* (f)		
	[A-T]/[A-T]	[A-T]/[T-T]	[A-T]/[A-C]	[A-T]	[T-T]	[A-C]
Spain (Madrid)						
Cases	68 (0.62)	17 (0.15)	20 (0.18)	173 (0.79)	27 (0.12)	2 (0.09)
Controls	62 (0.47)	50 (0.38)	10 (0.08)	184 (0.70)	66 (0.25)	14 (0.05)
USA (St Louis)						
Cases	104 (0.65)	24 (0.15)	27 (0.17)	259 (0.80)	33 (0.10)	30 (0.09)
Controls	96 (0.55)	42 (0.24)	23 (0.13)	258 (0.73)	60 (0.17)	32 (0.09)

$2 \times 2\ \chi^2$ test of [A-T]/[A-C] versus [A-T]/[T-T]: Spain, $P = 0.0001$; USA, $P = 0.050$.
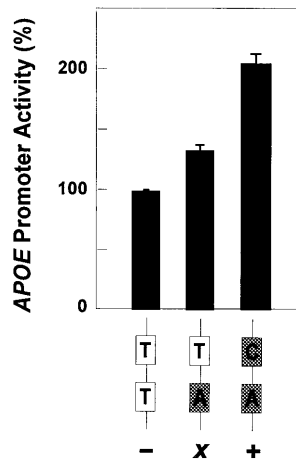$2 \times 2\ \chi^2$ test of [A-T]/[A-T] versus [A-T]/[T-T]: Spain, $P = 0.0003$; USA, $P = 0.027$.

Figure 1. Transcriptional activity of the [–491–427] haplotypes of the *APOE* promoter. Luciferase:β-galactosidase activity ratios for pXP2 constructs containing the [T-T] (–), [A-T] (*x*) and [A-C] (+) *APOE* haplotypes transfected into U87 astrocytoma cells are expressed as percentages of activity of the [T-T] construct. Data are means ± SEM of six determinations and are representative of five experiments.
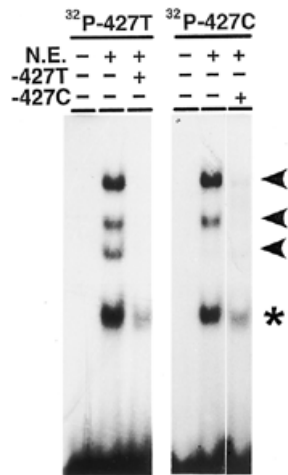

Figure 2. Protein binding of the –427T/C allelic forms of the *APOE* promoter. ^{32}P-labeled –427T and –427C probes were incubated with U87 nuclear extract (NE) and subjected to electrophoretic mobility shift assays. Competition experiments were performed by pre-incubating the nuclear extract with a 50-fold excess of the unlabeled oligonucleotide before addition of the probe. Arrows, specific shift bands; asterisk, non-specific band. Results are representative of four independent experiments.

Association between *APOE* promoter activity and risk for AD was then analyzed in the Spanish population stratified according to ApoE status (Table 3). A strong association between AD risk and promoter activity of the three predominant genotypes (*x*–, *xx* and *x+*) was observed in the Spanish non-ApoE4 individuals; taking as reference the *x*– individuals, the risk for AD was found to increase 6- and 18-fold in *xx* and *x+* individuals, respectively. Statistical significance of these data was evaluated by a logistic regression model. Analysis of the subgroup of non-E4 carriers showed a highly significant association between AD risk and promoter activity ($P < 10^{-10}$) and yielded predicted ORs of 4.3 (95% CI 2.9–6.3) and 18 (95% CI 8–40) for genotypes *xx* and *x+*, respectively (Fig. 3a).
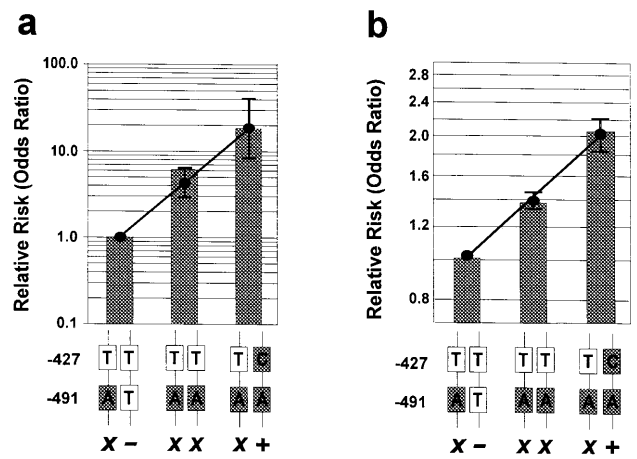

Figure 3. Association between risk for AD and *APOE* promoter genotypes in the (**a**) Spanish and (**b**) US non-ApoE4 subpopulations. The risks are represented on a log odds scale, taking as the reference the genotype *x*–, which bears the weakest expressing (–) haplotype. Bars represent the calculated ORs. The superimposed line represents the ORs estimated by the logistic regression model and error bars indicate the 95% CIs.

Finally, the hypothesis that AD risk increases with promoter transcriptional activity measured in the transfection assays was tested in the St Louis population. Although the association was less impressive than that encountered in the Spanish population, a similar dependence of OR for developing AD and promoter activity of the three predominant genotypes was detected in the non-E4 subpopulation (Table 3). The logistic regression analysis indicated a highly statistically significant association between AD risk and promoter activity ($P < 10^{-10}$), with ORs of 1.4 (95% CI 1.3–1.5) and 2.0 (95% CI 1.9–2.2) for genotypes *xx* and *x+*, respectively (Fig. 3b).

DISCUSSION

In this paper we report an association between polymorphisms in the proximal region of the *APOE* promoter and risk for AD. We have previously reported association of homozygosity for the –491A allele with risk for AD (7). Here we show that the –427C allele is also associated with risk for AD. Furthermore, combination of these two alleles into the [–491A–427C] haplotype generates a high risk for AD, in particular in the Spanish population, in individuals bearing no ApoE4 alleles. The same effect is observed in the St Louis sample, although the relative risk was lower than in the Spanish sample. The difference in relative risk is most probably attributable to differences in the frequencies of *APOE* promoter haplotypes in the two control populations, since the two AD samples show very similar frequencies of promoter haplotypes. It is well documented that population-based differences in ApoE4 allele frequency (9,10) give rise to population differences in relative risk for AD associated with this allele. Consistently, in our samples the OR associated with ApoE4 in the Spanish population (8.3) was more than double that in the St Louis sample (3.1) (7). Based on these data, we anticipate that there will be wide variation in the relative risk for AD associated with the *APOE* promoter polymorphism in different populations, as has already been noted for the ApoE4 allele (11). Dividing the sample on the basis of the presence or absence of ApoE4 revealed that the promoter polymorphisms and ApoE4 are independent risk factors. Indeed, the risk due to the promoter polymorphism was most prominent in individuals without ApoE4 alleles.

Table 3. *APOE* [–491–427] major genotype distribution in ApoE ε4 carriers and non-carriers; age at onset >60 years

ApoE4 status	Spain (Madrid): *n* (f)			USA (St Louis): *n* (f)		
	[A-T]/[A-T]	[A-T]/[T-T]	[A-T]/[A-C]	[A-T]/[A-T]	[A-T]/[T-T]	[A-T]/[A-C]
–/–						
Cases	21 (0.54)	3 (0.08)	11 (0.29)	42 (0.57)	16 (0.21)	14 (0.19)
Controls	52 (0.45)	45 (0.39)	9 (0.08)	67 (0.52)	35 (0.27)	15 (0.12)
–/+, +/+						
Cases	47 (0.66)	14 (0.20)	9 (0.13)	62 (0.71)	8 (0.09)	13 (0.14)
Controls	10 (0.59)	5 (0.29)	1 (0.06)	29 (0.60)	7 (0.14)	8 (0.17)

2×2 χ^2 test for [A-T]/[A-C] versus [A-T]/[T-T] in ApoE4 non-carriers: Spain, $P = 0.00001$; USA, $P > 0.05$ (not significant).
2×2 χ^2 test for [A-T]/[A-T] versus [A-T]/[T-T] in ApoE4 non-carriers: Spain, $P = 0.0024$; USA, $P > 0.05$ (not significant).
P-values for ApoE4 carriers were >0.05 (not significant) for both samples.

ApoE expression is regulated by complex interactions between developmental, hormonal and dietary factors (12–15). Regulation of *APOE* expression has been investigated thoroughly in hepatoma cells, where it has been shown that the elements required for efficient expression are located in the 5′ proximal region and in the first intron (16–18). More recently, we have demonstrated that this region also drives efficient expression of *APOE* in astrocytoma cells (19). The data presented in this report strongly suggest that allelic polymorphism in the transcriptional regulatory region of *APOE* may contribute to differences in the levels of ApoE synthesis by astrocytes. Specifically, our data indicate that the single base substitutions corresponding to the –491 and –427 *APOE* polymorphic sites produce differences in transcriptional activity of the *APOE* promoter due to differential binding of nuclear proteins. It is well documented that single nucleotide changes within a promoter region can dramatically affect transcriptional activity mediated by transcription factors (20–22).

The data presented here strongly suggest the existence of a direct correlation between risk for AD and *APOE* promoter activity in astrocytic cells, i.e. the higher the expression of ApoE, the higher the risk for AD. There is increasing evidence suggesting that increased levels of ApoE are associated with AD pathogenesis. Several studies have reported that ApoE levels are elevated in the brain and in plasma from AD patients (23–25). Intracellular accumulation of ApoE has also been correlated with neuronal cell death and intracellular Aβ stabilization (26). Recent studies have also demonstrated that the level of ApoE expression can modify both the cytotoxic effects of ApoE and the propensity of Aβ to deposit in the murine brain. ApoE at concentrations found in the cerebrospinal fluid protects neuronal cells from Aβ peptides, while higher ApoE concentrations could lead to neuronal cell death (27). Crossing *APOE* knockout mice with mice overexpressing a human mutant APP gene indicates that Aβ deposition correlates positively with ApoE dose and suggests that ApoE may facilitate Aβ peptide deposition *in vivo* (28).

Despite the evidence implicating ApoE4 as the single most important genetic determinant of susceptibility to late onset AD, its pathogenic role is not well understood. The amino acid substitutions associated with the E2, E3 and E4 ApoE isoforms lead to differences in the predicted three-dimensional structure of ApoE that are likely to alter the functional properties of the molecule. Several potential mechanisms of action of ApoE4 on AD risk have been proposed, based on the ApoE isoform-specific differences observed in several assays developed in brain cells (28–35). However, to date there is no adequate explanation for the variation in risk for AD associated with the different ApoE isoforms.

Based upon data presented here and elsewhere we suggest that there are two independent mechanisms by which AD risk can be modified in the *APOE* gene. The first involves variants in the coding region that alter the functional properties of the ApoE molecule, while the second involves promoter region variants that result in quantitative differences in ApoE expression. These two factors are associated with AD risk independently, i.e. each one can produce risk by itself, but may interact with each other resulting in an overall risk for individuals bearing both ApoE4 and high expressing *APOE* promoter forms that would depend on the relative contribution of each of these two risk factors.

In summary, our data strongly suggest that risk for AD associated with *APOE* promoter polymorphisms directly correlates with the transcriptional activity of the gene. Further, we speculate that increases in the local expression of ApoE, due to the *APOE* promoter polymorphisms, could lead to enhanced deposition of Aβ peptide in the brain, thus explaining the increased risk for AD. If this is correct, then drugs that either control ApoE synthesis or inhibit Aβ production or deposition may be useful in the treatment of AD.

MATERIALS AND METHODS

Study subjects

The test sample consisted of 132 healthy controls (age of onset 72 ± 7 years, mean $\pm$ SD) and 110 late onset sporadic AD cases (68 ± 5 years) from the Madrid region of Spain, recruited from the Neurology Service of the Hospital La Paz of Madrid. The replication sample was composed of 175 healthy controls (78 ± 9 years) and 160 AD (36,37) cases (75 ± 8 years) from the St Louis metropolitan region of the USA, recruited through the Alzheimer's Disease Research Center at Washington University. Both samples included only individuals of Caucasian origin.

Genotyping of –491A/T, –427T/C and ApoE polymorphisms

The *APOE* region –512 to –285 was amplified by nested PCR using a mismatched primer to generate a *Dra*I site specific for the –491A allele. Briefly, PCR was used to amplify a 1423 bp fragment

of the transcriptional regulatory region, spanning –1017 to +406 relative to the transcriptional start site (16). Primers 5′-CAAGGT-CACACAGCTGGCAAC-3′ and 5′-TCCAATCGACGGC-TAGCTACC-3′ were used to amplify the whole transcriptional regulatory region. A nested PCR was used because we had difficulty in reliably amplifying the –512 to –285 fragment directly from genomic DNA. The 1423 bp PCR product was therefore used as template in the following reaction. The forward (mismatched) primer was 5′-TGTTGGCCAGGCTGGT<u>TTT</u>AA-3′, the reverse primer 5′-CCTCCTTTCCTGACCCTGTCC-3′. To analyze the –491 and–427 genotypes, as well as the [–491–427] haplotype, the product was digested with *Dra*I and *Alu*I restriction enzymes together. ApoE isoform genotyping was performed as described (38,39).

Transfection assays

The 5′ region between positions –1017 and +406 of the *APOE* gene was cloned into the pXP2-luciferase reporter plasmid as described (19). PCR-based site-directed mutagenesis was used to replace the A by a T at –491 and the T by a C at –427 using a modification of a described method (40). The constructs were transfected into U87 human astrocytoma cells and luciferase and β-galactosidase activities of the extracts were determined as described (19).

Electrophoretic mobility shift assays (EMSA)

EMSA of oligonucleotide probes by U87 nuclear extracts was performed as described (5,7). The oligonucleotides 5′-GCT-GGTCTCAA(**A/T**)CTCCTGACCTTAA-3′ and 5′-ACAGGC-GTGAGC(**T/C**)ACCGCCCCCAGC-3′ were used to study nuclear protein binding to the –491 and –427 sites, respectively.

Statistical analysis

Genotype and allele/haplotype distributions were analyzed by 2 × 2 χ^2 test. Relative risks (ORs) and 95% CIs were calculated as described (41). A logistic regression model was used to estimate relative risks for AD of the major *APOE* promoter genotypes as a function of the transcriptional activity.

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ABBREVIATIONS

AD, Alzheimer's disease; ApoE, apolipoprotein E; *APOE*, apolipoprotein E gene; APP, β-amyloid precursor protein; CI, confidence interval; OR, odds ratio.

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