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ORIGINAL ARTICLE

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Although possession of the ϵ4 allele of the apolipoprotein E gene appears to be an important biological marker for Alzheimer's disease (AD) susceptibility, strong evidence indicates that at least one additional risk gene exists on chromosome 12.

Here, we describe an association of the 3′-UTR +1073 C/T polymorphism of the *OLR1* (oxidised LDL receptor 1) on chromosome 12 with AD in French sporadic (589 cases and 663 controls) and American familial (230 affected sibs and 143 unaffected sibs) populations. The age and sex adjusted odds ratio between the CC+CT genotypes versus the TT genotypes was 1.56 (p=0.001) in the French sample and 1.92 (p=0.02) in the American sample. Furthermore, we have discovered a new T/A polymorphism two bases upstream of the +1073 C/T polymorphism. This +1071 T/A polymorphism was not associated with the disease, although it may weakly modulate the impact of the +1073 C/T polymorphism.

Using 3′-UTR sequence probes, we have observed specific DNA protein binding with nuclear proteins from lymphocyte, astrocytoma, and neuroblastoma cell lines, but not from the microglia cell line. This binding was modified by both the +1071 T/A and +1073 C/T polymorphisms. In addition, a trend was observed between the presence or absence of the +1073 C allele and the level of astrocytic activation in the brain of AD cases. However, $A\beta_{40}$, $A\beta_{42}$, Aβ total, and Tau loads or the level of microglial cell activation were not modulated by the 3′-UTR *OLR1* polymorphisms. Finally, we assessed the impact of these polymorphisms on the level of *OLR1* expression in lymphocytes from AD cases compared with controls. The *OLR1* expression was significantly lower in AD cases bearing the CC and CT genotypes compared with controls with the same genotypes. In conclusion, our data suggest that genetic variation in the *OLR1* gene may modify the risk of AD.

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that occurs predominantly in later life. The main pathological features of AD are characterised by neurofibrillary tangles and senile plaques caused by the progressive deposition of Aβ peptides in the brain, composed mainly of 39-43 peptides generated by proteolytic cleavage of the Aβ precursor protein (APP).

Genetic variations in four genes have been directly linked to the pathogenesis of AD. Mutations in three genes coding for the amyloid precursor protein (*APP*), presenilin 1 (*PS-1*), and presenilin 2 (*PS-2*) account for most cases of early onset, autosomal dominant familial AD (FAD), but only for 2% of all the AD cases.[1 2] The genetics of the late onset form of the disease is far more complex and only the ϵ4 allele of the apolipoprotein E (*APOE*) gene has so far been identified as playing a major role in the causation of sporadic cases.[3]

Although all these genes are known or suspected to be involved in the "amyloid cascade" either by increasing Aβ production or deposition or by enhancing Aβ toxicity,[4] *APOE* differs in several aspects from these other genes and, until now, is the only gene known to be involved in the lipid pathway.[5] A growing body of evidence suggests that lipid metabolism and high cholesterol levels might be linked to AD.[6] Epidemiological studies indicate that people treated with inhibitors of cholesterol biosynthesis, such as statins, may have a reduced risk of developing AD.[7] Interestingly, numerous in vitro and in vivo studies have shown that cholesterol modulates APP processing and affects APP mRNA expression, whereas Simvastatin has been reported to reduce strongly the level of both $A\beta_{40}$ and $A\beta_{42}$ peptides.[6 8 9] Finally, the *APOE* ϵ4 allele has been correlated with high cholesterol level.[10] ApoE is a lipid and cholesterol transport protein responsible for the efflux of cholesterol from neurones and is able to bind Aβ peptides to form stable complexes both in vitro and in vivo.[11 12] Altogether, these data suggest a link between cholesterol and AD, and logically lead us to hypothesise that genes implicated in lipid metabolism may be good candidates for AD.

It is generally agreed that the best way to study complex disorders, such as AD, is by a combination of association studies and allele sharing linkage methods in pairs of affected sibs or other relative pairs. Particularly, genome scan studies provide strong evidence that at least one risk gene exists on chromosome 12.[13–15] To date, three candidate genes on this chromosome have been proposed to be associated with AD. Two of them, the *LRP1* and *LBP1/CP2/LSF* genes are located on the long arm of chromosome 12.[16 17] However, only the latter has consistently shown a modest effect in independent data sets.[18 19] The third gene, the α2-macroglobulin (*A2M*) gene, is located on the short arm of chromosome 12.[20] However, the association of this gene with the risk of developing AD is still controversial and a recent meta-analysis has excluded it as a genetic determinant for sporadic AD.[21]

There are, however, a number of other candidate genes in this region. One of these candidate genes codes for the lectin type oxidised low density lipoprotein receptor 1 (*OLR1*), which is located in close proximity to *A2M*. Recently, a polymorphism in the 3′ untranslated region (UTR) of the *OLR1* gene, +1073 C/T, has been reported to be associated with AD after stratification by *APOE* genotype.[22] In this present study, we confirm the association of this polymorphism with AD, but independently of the *APOE* genotype in two different case-control samples. Furthermore, we describe a new polymorphism in the

3′-UTR, +1071 T/A, and its synergistic association with AD in conjunction with the +1073 C/T polymorphism. We also report that these two *OLR1* 3′-UTR polymorphisms affect the binding of nuclear proteins.

MATERIALS AND METHODS

Population description

The French AD and control samples were white (AD cases n=589, mean age = 72.3 (SD 7.2) years, mean age at onset = 69.4 (SD 7.4) years, 39.5% of men; controls n= 663, mean age = 72.5 (SD 7.9) years, 36% of men). Early age at onset was defined as ⩽65 years. Diagnosis of probable AD was established according to the DSM-III-R and NINCDS-ADRDA criteria. The white controls were defined as subjects without DMS-III-R dementia criteria and with integrity of their cognitive functions. Each person or next of kin gave informed consent.

The family sample was obtained from the Indiana Alzheimer's Disease Center (IADC) National Cell Repository that included 373 discordant sibs (230 affected, 143 unaffected) from 82 families. All affected sibs had late onset AD with mean age 74.2 (SD 6.0) years and mean age at onset 73.5 (SD 5.4) years, and 34.3% were male. Unaffected sibs had mean age 79.1 (SD 6.9) and 42.0% were male.

Brain samples

Brains were obtained at necropsy from 113 patients with early and late onset sporadic AD (mean age at onset 64.5 (SD 10.5) years, mean age at death 72.9 (SD 9.8) years, 49% were male). Genomic DNA was extracted from frozen brain tissue by standard methods and the *ORL1* 3′-UTR polymorphisms and the *APOE* genotype determined by PCR as described in the genotyping section.

The proportion of tissue area occupied by $A\beta_{40}$, $A\beta_{42}$, and total Aβ were quantified in immunohistochemically stained section from brodmann area 8/9 of the frontal cortex, as previously reported.[23] Tau load was determined in 86 samples after immunostaining for phosphorylated Tau using a standard procedure using monoclonal antibody AT8 (Innogenetics, Belgium), as primary antibody.[24] Ferritin immunostaining, as a tissue marker of activated microglia, was performed on 72 cases using a standard procedure, incubation in primary antibody (Sigma, UK) being performed overnight at 4°C at a dilution of 1:750. Sites of ferritin immunoreaction were visualised by 3,3 diaminobenzidene (DAB). Tau and microglial cell quantification was performed as described elsewhere.[24] Astrocyte activation level was assessed by GFAP immunohistochemistry. Primary GFAP antibody (Sigma) was used at 1:500 and the level of staining was rated semi-quantitatively according to 0=absent, 1=few, 2=moderate, 3= high, 4=very high.[24] All sections exhibited at least a few activated astrocytes and categories 1 and 2 were pooled for analyses.

Sequencing and genotyping

The 3′-UTR sequence was amplified as described by Luedecking-Zimmer *et al*.[22] PCR products were directly sequenced using the *Taq* dye Terminator sequencing kit (Perkin Elmer Biosystems, Foster City, CA).

The *APOE* genotypes were identified by PCR followed by *Hha*I digestion, as slightly modified from the methods of Hixson and Vernier.[26]

The 3′-UTR genotypes were determined by two methods. (1) An amplification of a 156 bp fragment using mismatched reverse primer 5′-ACAAGCTAGGTGAAATAATACCG-3′ and reverse primer 5′-CTATTCTTTGTCACTTGGG-3′ was performed. The +1071 A/T genotypes were determined by *Alu*I digestion and the +1073 C/T genotypes by *Msp*I digestion (site created by the reverse mismatched primer) after separation on a 3% agarose gel. (2) The method described by Luedecking-Zimmer *et al*[22] was used for the +1073 C/T genotyping using *Dpn*II digestion while the amplicon used for sequencing was digested by *Alu*I for the +1071 A/T genotyping. The genotyping on the French and brain samples was done in Lille, while the genotyping on the American sample was done in Pittsburgh. Approximately 10% of the genotyping performed in France was done using the two PCR methods described above and no discrepancy was observed.

Electrophoretic mobility shift assays

Cytoplasmic and nuclear extracts from lymphocyte (healthy subject) microglia cell line (CHME-5), astrocytoma (STTG-1), or neuroblastoma (Kelly) were prepared according to described methods.[26] Single stranded oligonucleotides (5′-TTTTGATTCTAGCTATCTGTATTATTTCAC-3′) were end labelled with digoxigenin, annealed to complementary oligomer, and incubated for 20 minutes at room temperature with cytoplasmic or nuclear extract (5 μg). Proteins were added to a final volume of 20 ml of a mixture described elsewhere.[17] The complexes were separated on a 5% non-denaturing polyacrylamide gel and semi-dry electrophoretic transfer was performed from gels to nitrocellulose membranes. Detection was as described by the supplier (Roche Diagnostics, Meylan, France).

Semi-quantitative RT-PCR assays

Purified lymphocytes from fresh blood of 20 AD cases (81.8 (SD 5.8) years, 25% of men) and 39 controls (80.3 (SD 6.5) years, 29.3% of men) were cultivated for 72 hours in the presence of 0.1% phytohaemagglutinin. Following harvesting, total RNA extraction was performed using RNeasy Mini kit (Qiagen) associated with systematic DNase treatment. Quality of the total RNA was assessed using Agilent technology indicating that the ratio of the ribosomal RNA 28S and 18S was systematically superior to 1.8. Retrotranscription was realised. From 30 ng of cDNA/RNA, real time PCR using *Taq*man technology was performed to coamplify cDNA from the *ORL1* and β-actin genes as described by the supplier (Applied Biosystems).

Statistical analyses

The SAS software release 6.11 was used (SAS Insitute, Cary, NC). Univariate analysis was performed using Pearson's χ^2 test. In the multivariate analysis, we coded the genotype of the *ORL1* polymorphisms by dichotomising genotypes into the presence or absence of a C allele for the +1073 C/T (CC+CT versus TT) and of an allele for the +1071 T/A (AA+AT/TT). The effect of these variables on the disease were assessed by a multiple logistic regression model adjusted for age and gender. Interaction between *APOE* and 3′-UTR polymorphisms were tested by logistic regression as well as tests for linear trends.

Pairwise linkage disequilibrium coefficients were estimated in the control samples. Extended haplotype frequencies of the two markers were estimated on collapsed data using the myriad haplotype algorithm described by McLean *et al*.[27]

Comparison of the Aβ and Tau loads, microglia and astrocyte activation, *ORL1* expression according to AD status or 3′-UTR *OLR1* genotypes was performed using non-parametric analyses. Results from *OLR1* mRNA semi-quantification was log transformed to normalise their distributions.

RESULTS

Upon sequencing the 3′-UTR of the *ORL1* gene, we found a new T/A polymorphism, two bases upstream of the previously reported +1073 C/T polymorphism (numbering from the NM_002543 sequence). We first analysed the impact of these two polymorphisms on the risk of developing AD in a French

Table 1 Allelic and genotypic distribution of the *ORL1* 3′-UTR polymorphisms in the French (A) and American (B) samples

(A) France		Allele distribution (%)		Genotype distribution (%)		
+1073 C/T	No	C	T	CC	CT	TT*
AD cases	598	658 (0.55)	538 (0.45)	178 (0.30)	302 (0.50)	118 (0.20)
Control	663	638 (0.48)	688 (0.52)	158 (0.23)	322 (0.49)	183 (0.28)
+1071 T/A	No	T	A	TT	TA	AA†
AD cases	598	1044 (0.87)	152 (0.13)	450 (0.752)	144 (0.241)	4 (0.007)
Control	663	1180 (0.89)	146 (0.11)	525 (0.792)	130 (0.196)	8 (0.012)
(B) USA		**Allele distribution (%)**		**Genotype distribution (%)**		
+1073 C/T	No	C	T	CC	CT	TT‡
AD cases	230	277 (0.60)	183 (0.40)	90 (0.39)	97 (0.42)	43 (0.19)
Control	143	161 (0.56	125 (0.44)	52 (0.36)	57 (0.40)	34 (0.24)
+1071 T/A	No	T	A	TT	TA	AA§
AD cases	225	397 (0.88)	53 (0.12)	176 (0.78)	45 (0.20)	4 (0.02)
Control	141	252 (0.89)	30 (0.11)	115 (0.82)	22 (0.15)	4 (0.03)

*OR (CC+CT versus TT)=1.56, 95% CI 1.19 to 2.04, p=0.001, adjusted for age and gender.
†OR (AA+AT versus TT)=1.25, 95% CI 0.96 to 1.63, p=0.11, adjusted for age and gender.
‡OR (CC+CT versus TT)=1.92, 95% CI 1.10 to 3.35, p=0.02, adjusted for age and gender.
§OR (AA+AT versus TT)=1.42, 95% CI 0.81 to 2.51, p=0.22, adjusted for age and gender.

sample comprising 598 sporadic AD cases and 663 controls. The genotype distributions for both polymorphisms were in Hardy-Weinberg equilibrium (table 1). A statistically significant association with the risk of AD was observed only with the +1073 C/T polymorphism. The frequency of the +1073 C allele was significantly higher in AD cases than controls (55% *v* 48%, p=0.0005). Subjects bearing at least one +1073 C allele had an increased risk for AD (OR=1.56, 95% CI 1.19 to 2.04, p<0.001). No significant increased risk was observed for subjects bearing at least one +1071 A allele (OR=1.25, 95% CI 0.96 to 1.63, p=0.11). No significant interaction was detected between gender, *APOE* status, and the 3′-UTR polymorphisms.

The two polymorphisms were in near complete linkage disequilibrium in the French sample (99.5%, p<0.001). Three common haplotypes, T-T, T-C, and A-C, were observed. The fourth haplotype, A-T, was observed in only three controls (0.3%) (table 2A). Two site genotypes based on three common haplotypes were assigned to each subject and haplotype frequencies compared between AD cases and controls (table 2A). The estimated haplotype distribution was significantly different between AD cases and controls. Compared to the T-T/T-T combined genotype, subjects carrying the other two haplotypes combination were at an increased risk of developing AD (table 2B). The strongest effect was observed for subjects bearing T-C/A-C, suggesting a potential but weak interaction between both polymorphisms.

The association seen in the French sporadic AD sample was confirmed in a family based sample from the USA comprising 373 discordant sibs (230 affected, 143 unaffected). As observed in the French sample, subjects bearing at least one +1073 C allele had an increased risk for AD (OR=1.92, 95% CI 1.10 to 3.35, p=0.02) and this association was independent of *APOE*. Similarly, no significant increased risk was observed for subjects bearing at least one +1071 A allele (OR=1.42, 95% CI 0.81 to 2.51, p=0.22). Haplotype analysis also showed similar results in the USA sample to those seen in the French sample (table 2).

We next considered whether these two polymorphisms have biologically relevant roles consistent with modifying the risk for AD. We used electrophoretic mobility shift assays to determine if the DNA sequence spanning both polymorphisms interacts with cytoplasmic or nuclear protein extracts from

Table 2 (A) Haplotype distribution in the control and AD populations (B) risk of developing AD according to the haplotype genotypes

(A)	Estimated haplotype distribution (%)			
	France		USA	
Haplotype	Controls	AD cases	Controls	AD cases
1 TAGCTATCTGTATTA	687 (51.9)	538 (45.0)	125 (44.3)	176 (39.1)
2 TAGCTACCTGTATTA	494 (37.0)	506 (42.3)	127 (45.0)	221 (49.1)
3 TAGCAACCTGTATTA	142 (10.8)	152 (12.7)	30 (10.6)	53 (11.8)
4 TAGCAATCTGTATTA	3 (0.2)	–	–	–
p	0.0004		0.38	

(B)	France		USA	
Haplotype combination	Adjusted OR (Whole)	p	Adjusted OR (Whole)	p
11	Reference		Reference	
12	1.43 (1.07 to 1.92)	0.02	1.78 (0.95 to 3.34)	0.07
13	1.39 (0.92 to 2.10)	0.12	1.64 (0.52 to 5.19)	0.40
22	1.56 (1.08 to 2.27)	0.02	1.87 (0.94 to 3.73)	0.08
23*	2.12 (1.41 to 3.19)	0.0003	2.32 (1.03 to 5.23)	0.04
Test for trends		0.001		0.16

All ORs are adjusted for age and gender.
*The 33 genotype was not included in this table because of the small number of subjects bearing this genotype.

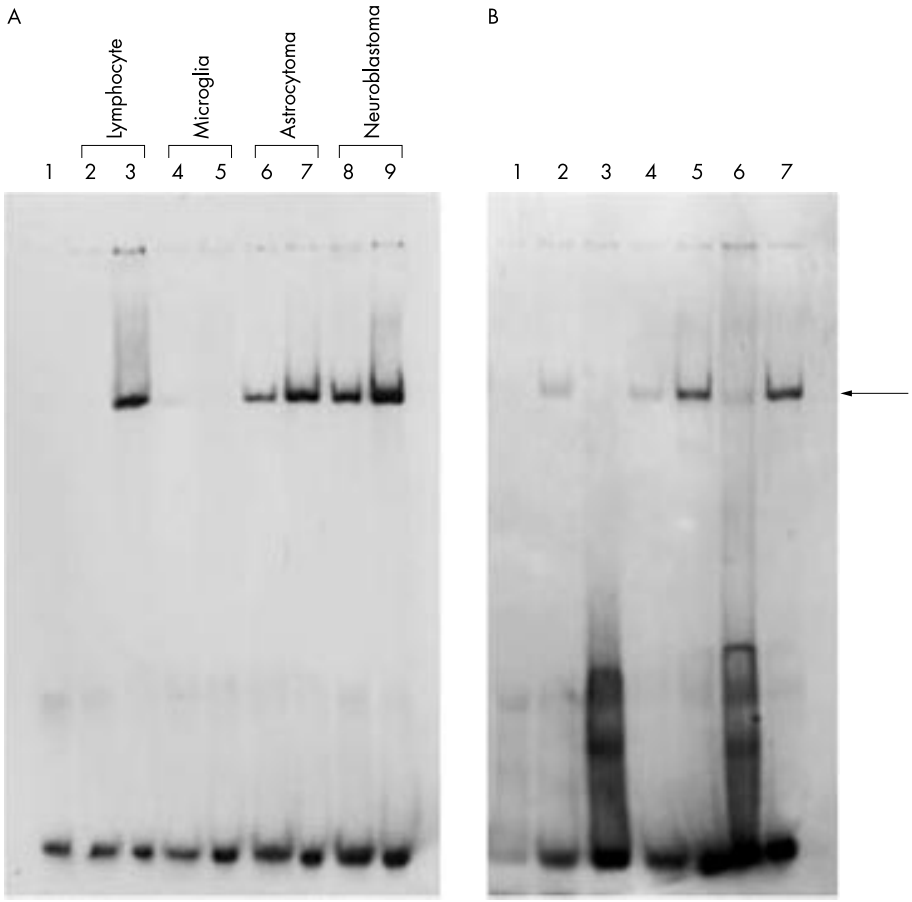

Figure 1 The labelled 3′-UTR TT haplotype probes were incubated (A) with cytoplasmic (lanes 2, 4, 6, 8) or nuclear (lane 3, 5, 7, 9) protein extracts from lymphocyte, CHME-5 microglia, STTG1 astrocytoma, or Kelly neuroblastoma cell lines (negative control, lane 1) and (B) with cytoplasmic (lane 2) or nuclear proteins alone (lane 5), unlabelled 3′-UTR T-T haplotype oligo (lanes 3, 6), or scramble oligo (lanes 4, 7). Proteins were extracted from neuroblastoma (negative control, lane 1).

different cell lines. DNA-protein complexes were observed from lymphocyte, astrocytoma, and neuroblastoma extracts, but not from the microglial cell extract. The T-T haplotype probe of the 3′-UTR *OLR1* polymorphisms showed a weaker protein-DNA complex formation with cytoplasmic proteins as compared to nuclear proteins (fig 1A). The 3′-UTR *OLR1* probe binding was competed by an unlabelled 3′-UTR *OLR1* oligonucleotide, supporting the specificity of nuclear protein binding (fig 1B). We next compared the affinity of these proteins for oligomers containing different haplotype combinations. We found that the A-T haplotype displayed an average 1.8-fold higher affinity than the T-T haplotype under equilibrium conditions (fig 2A), suggesting that the +1071 T/A polymorphism weakly modifies the affinity of nuclear proteins for this 3′-UTR sequence. On the other hand, the T-C haplotype displayed an average six-fold higher affinity than the T-T haplotype under equilibrium conditions (fig 2B), suggesting that the +1073 C/T polymorphism strongly modulates the affinity of nuclear proteins with this 3′-UTR sequence. However, we did not detect a difference of binding affinity when comparing the T-C and A-C haplotype sequences (fig 2C). This may not be surprising, as the presence of the +1073C allele, which is associated with higher binding affinity than the +1071A allele, in both haplotypes (T-C and A-C) is probably obscuring the small affinity difference between the +1071 A and +1071 T allele. This observation is consistent with our epidemiological data suggesting at best a weak interaction between both polymorphisms.

In order to characterise better the potential impact of these polymorphisms on the aetiology of the disease, we examined the effect of these two polymorphisms on Aβ and Tau loads and astrocyte and microglial cell activation in AD brains. No impact of this polymorphism was observed on Aβ, Tau loads, or microglial cell activation (data not shown) but a possible association of the +1073 C/T polymorphism with astrocyte activation was observed, the +1073 C allele being associated with a higher level of activation (table 3). The low frequency of the +1071 T/A polymorphism did not allow meaningful statistical analysis (data not shown).

We finally investigated whether these differences were reflected by altered expression of the *OLR1* gene. Using a semi-quantitative RT-PCR assay, we estimated the ratio between the amount of the *OLR1* and β-actin mRNA from lymphocytes of control, AD, and young subjects. The level of expression of the *OLR1* gene in relation to the β-actin gene was significantly lower in AD cases compared with controls ($p=0.009$) (table 4) and this difference was confined to subjects bearing the CC and CT genotypes ($p=0.009$). These data indicate that the +1073 C allele may be functional by lowering the *ORL1* expression in AD lymphocytes more than controls. The low frequency of the +1071 T/A polymorphism did not allow meaningful statistical analysis (table 4).

DISCUSSION

The 3′-UTR +1073 C/T polymorphism has been shown to modify the risk of sporadic AD in an *APOE* dependent fashion.[22] In this paper, we report the association of the 3′-UTR +1073 C/T polymorphism with the risk of AD independent of *APOE* status in two different samples comprising French sporadic and American familial AD cases. We also describe a new polymorphism, +1071 T/A, located two bases upstream of the

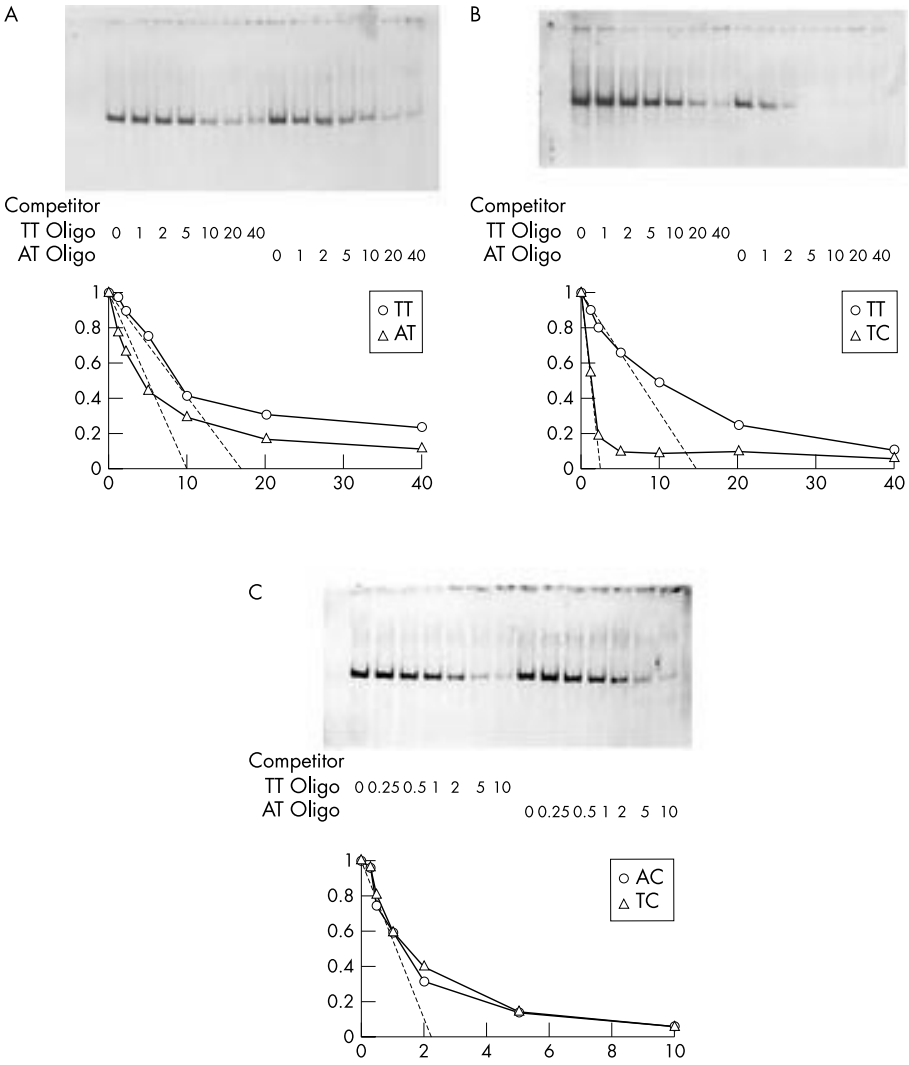

Figure 2 Modulation of the binding of nuclear proteins extracted from neuroblastoma by the +1071 T/A and +1073 C/T polymorphisms. Lanes show decreasing complex formation owing to increasing competition for each allele. (A) Competition of binding with increasing concentration of excess of unlabelled oligo under equilibrium conditions for the haplotypes T-T and A-T. The graph represents the relative binding of the haplotype T-T compared to the haplotype A-T. The slopes of the dashed lines that represent the initial linear phases of competition are described by the equation y=–0.059x+1.00 for the haplotypes T-T and y=–0.103x+1.00 for the haplotype A-T. (B) Competition of binding with increasing concentration of excess of unlabelled oligo under equilibrium conditions for the haplotypes T-T and T-C. The graph represents the relative binding of the haplotype T-T compared to the haplotype T-C. The slopes of the dashed lines that represent the initial linear phases of competition are described by the equation y=–0.067x+1.00 for the haplotypes T-T and y=–0.403x+1.00 for the haplotype T-C. (C) Competition of binding with increasing concentration of excess of unlabelled oligo under equilibrium conditions for the haplotypes A-C and T-C. The graph represents the relative binding of the haplotype A-C compared to the haplotype T-C. The slopes of the dashed lines that represent the initial linear phases of competition are described by the equation y=–0.381x+1.00 for the haplotypes A-C and y=–0.393x+1.00 for the haplotype T-C.

+1073 C/T polymorphism. Although the latter polymorphism was not associated with AD by itself, the two site haplotype analysis suggested a potential weak combined effect of these two polymorphisms on the risk of AD.

The relevance of the *OLR1* 3′-UTR polymorphisms, especially the +1073 C/T polymorphism, to the risk of AD is highlighted by the functional experiments we performed. Indeed, we show that the 3′-UTR sequence encompassing the two polymorphisms binds differentially to regulatory proteins; the

Table 3 Level of astrocyte activation according to the *ORL1* +1073 C/T polymorphism in brains of AD cases

		Allele distribution		Genotype distribution	
Level of activation*	No	T allele	C allele	TT	CT+CC
1, 2	31	29 (46.8)	33 (53.2)	7 (0.23)	24 (0.77)
3	23	18 (39.1)	28 (60.9)	2 (0.09)	21 (0.91)
4	5	1 (10.0)	9 (90.0)	0 (–)	5 (1.00)
Test for trends		0.05		0.09	

*Astrocyte activation was rated semiquantitatively as: 1=few, 2=moderate, 3=high, and 4=very high.

Table 4 Level of *OLR1* mRNA in lymphocytes from AD cases and elderly controls according to the 3′-UTR polymorphisms

Level of *ORL1* mRNA (arbitrary units)	No	Controls	No	AD cases
Whole population	39	1.67 (0.40 to 7.03)	20	0.61 (0.13 to 2.81)*
+1073 C/T genotype				
TT	9	0.94 (0.21 to 4.16)	5	0.90 (0.12 to 2.81)†
CC+CT	25	1.96 (0.43 to 8.91)	15	0.53 (0.13 to 2.14)‡
3′-UTR T/A genotype				
TT	24	1.72 (0.34 to 8.79)	18	0.66 (0.14 to 3.24)†
AT	10	1.39 (0.39 to 4.98)	2	0.29 (0.17 to 0.48)†

*AD cases versus controls: p=0.009.
†AD cases versus controls: p=NS.
‡AD cases versus controls: p=0.009.

+1071 A and +1073 C alleles bind with higher affinity compared to the +1071 T and +1073 T alleles, respectively. Furthermore, the highest level of astrocyte activation in AD brains may be associated with the presence of the +1073 C allele. Finally, the +1073 C allele may be associated with a specific decrease of *OLR1* expression in lymphocytes from AD cases.

In addition to these positive associations, there are, however, some caveats that need to be considered since no effects of the 3′-UTR polymorphisms were observed on Aβ and Tau loads. Similarly, microglial cell activation seems not to be modulated by these polymorphisms. However, this latter observation might be expected, as no DNA-protein complex was observed using protein extract from a microglial cell line. On the other hand, the 3′-UTR sequence formed a DNA-protein complex with the astrocytic proteins and the astrocytic activation was affected by the +1073 C/T polymorphism. These data suggest that *OLR1* may act through a specific local mechanism involving astrocytes.

Another important point is the significant reduction of *OLR1* expression in lymphocytes (−362%) in AD cases compared to controls indicating that control of the expression of this gene is potentially important for the development of AD. However, a quantification from brain tissue appears necessary since the expression of *OLR1* is about five-fold higher in different parts of the brain than in peripheral lymphocytes.[28] Furthermore, we cannot exclude the possibility that the potential effect of the 3′-UTR polymorphisms on *OLR1* expression is specific to brain tissue, even restricted to one cell type, such as the astrocyte.

The exact mechanism by which *OLR1* may affect the risk of AD is not clear as yet. *OLR1* is abundantly expressed in the human central nervous system (CNS),[28] but its physiological role has not been evaluated. Despite the existence of the blood-brain barrier, lipoprotein particles have been shown also to be present in the cerebrospinal fluid (CSF). Although some of these protein components may filter through the barrier from the vascular compartment, experimental evidence indicates that these particles originate from nervous tissue.[29] Furthermore, it has been shown that lipoprotein particles can be isolated from the media of astrocytic cultures.[30] CSF and CNS lipoproteins seem to be vulnerable to oxidative modifications.[31] This is of particular relevance to Alzheimer's disease since reactive oxygen species are abundantly produced following chronic inflammation.[32] Lipoprotein oxidation may disrupt several mechanisms leading or facilitating the disease. (1) Oxidised LDL (oxLDL) or oxidised HDL (oxHDL) may directly induce neuronal death in vitro. Coapplication of oxLDL or oxHDL with amyloid peptide, which enhances oxidative stress, resulted in increased neuronal death.[33 34] (2) Aβ peptides are associated with lipoproteins secreted by cultured astrocytes. We may suppose oxidation of these lipoproteins may modify their ability to bind amyloid fibrils, disrupting a potential mechanism of Aβ clearance.[35] (3) The growth and maintenance of an elaborate neurite network requires directional lipid transport; alterations in this lipid transport might be detrimental to neuronal integrity. It has been proposed that neurones must import glial cell derived cholesterol via lipoproteins to form numerous and efficient synaptic connections.[36] In this way, the main phase of synaptogenesis starts synchronously after glial cell differentiation throughout the CNS.[37] Neurological symptoms of AD may be associated with defective cholesterol and lipoprotein metabolism.[6] Disruption of this metabolism may directly result from lipoprotein oxidation or from interaction with Aβ peptides. Indeed, it has recently been suggested that Aβ peptides may directly regulate cholesterol transport and homeostasis via lipoproteins and overproduction of Aβ or high cholesterol may block cholesterol trafficking.[38] Oxidation of lipoproteins may accentuate this mechanism.

In addition to being functionally relevant to affecting the risk of AD, *OLR1* is also a positional candidate gene for AD based on the linkage mapping data in white[13–15 39 40] and non-white[41] families. Indeed, a repeat polymorphism in the 3′-UTR of *OLR1* has shown the strongest evidence of linkage on chromosome 12 with AD.[40] Recently, although Blacker *et al*[42] have reported the absence of linkage on chromosome 12 in the full NIMH AD Genetics Initiative families (411 white, 26 non-white), they continue to see strong association with A2M, which is located in close proximity to *OLR1* on chromosome 12. Furthermore, significant evidence of linkage on chromosome 12 has been reported in 230 of the NIMH families,[39 40] Thus, our current *OLR1* association data in conjunction with earlier OLR1 association[22] and linkage[13–15 39–41] data strongly suggest that the *OLR1* gene may directly affect the risk of AD. This study, however, does not exclude the possibility that the effect observed here could be attributable to linkage disequilibrium with a functional mutation in the promoter or 5′-UTR region of *OLR1* or to linkage disequilibrium with functional mutations in nearby genes. Additional analyses of the *OLR1* gene or nearby genes may help to identify the chromosome 12 AD gene.

In conclusion, our data suggest that the *OLR1* gene may be a risk factor for AD. This observation, combined with other studies showing that other genes coding for the LDL receptor family may be genetic determinants of AD, underlines the importance of cholesterol trafficking in the aetiology of AD.

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REFERENCES

1 **Cruts M**, van Duijn CM, Backhovens H, Van den Broeck M, Wehnert A, Serneels S, Sherrington R, Hutton M, Hardy J, St George-Hyslop PH, Hofman A, Van Broeckhoven C. Estimation of the genetic contribution of presenilin-1 and -2 mutations in a population-based study of presenile Alzheimer disease. *Hum Mol Genet* 1998;**7**:43-51.
2 **Campion D**, Dumanchin C, Hannequin D, Dubois B, Belliard S, Puel M, Thomas-Anterion C, Michon A, Martin C, Charbonnier F, Raux G, Camuzat A, Penet C, Mesnage V, Martinez M, Clerget-Darpoux F, Brice A, Frebourg T. Early-onset autosomal dominant Alzheimer disease: prevalence, genetic heterogeneity, and mutation spectrum. *Am J Hum Genet* 1999;**65**:664-70.
3 **Farrer LA**, Cupples LA, Haines JL, Hyman B, Kukull WA, Mayeux R, Myers RH, Pericak-Vance MA, Risch N, van Duijn CM. Effects of age, sex, and ethnicity on the association between apolipoprotein E genotype and Alzheimer disease. A meta-analysis. APOE and Alzheimer Disease Meta Analysis Consortium. *JAMA* 1997;**278**:1349-56.
4 **Hardy J**. Amyloid, the presenilins and Alzheimer's disease. *Trends Neurosci*. 1997;**20**:154-9.
5 **Fagan AM**, Holtzman DM. Astrocyte lipoproteins, effects of apoE on neuronal function, and role of apoE in amyloid-beta deposition in vivo. *Microsc Res Tech* 2001;**50**:297-304.
6 **Hartmann T**. Cholesterol, A beta and Alzheimer's disease. *Trends Neurosci* 2001;**24**:S45-8.
7 **Jick H**, Zornberg GL, Jick SS, Seshadri S, Drachman DA. Statins and the risk of dementia. *Lancet* 2000;**356**:1627-31.
8 **Howland DS**, Trusko SP, Savage MJ, Reaume AG, Lang DM, Hirsch JD, Maeda N, Siman R, Greenberg BD, Scott RW, Flood DG. Modulation of secreted beta-amyloid precursor protein and amyloid beta-peptide in brain by cholesterol. *J Biol Chem* 1998;**273**:16576-82.
9 **Fassbender K**, Simons M, Bergmann C, Stroick M, Lutjohann D, Keller P, Runz H, Kuhl S, Bertsch T, von Bergmann K, Hennerici M, Beyreuther K, Hartmann T. Simvastatin strongly reduces levels of Alzheimer's disease beta -amyloid peptides Abeta 42 and Abeta 40 in vitro and in vivo. *Proc Natl Acad Sci USA* 2001;**98**:5856-61.
10 **Luc G**, Bard JM, Arveiler D, Evans A, Cambou JP, Bingham A, Amouyel P, Schaffer P, Ruidavets JB, Cambien F. Impact of apolipoprotein E polymorphism on lipoproteins and risk of myocardial infarction. The ECTIM Study. *Arterioscler Thromb* 1994;**14**:1412-19.
11 **Pillot T**, Goethals M, Najib J, Labeur C, Lins L, Chambaz J, Brasseur R, Vandekerckhove J, Rosseneu M. Beta-amyloid peptide interacts specifically with the carboxy-terminal domain of human apolipoprotein E: relevance to Alzheimer's disease. *J Neurochem* 1999;**72**:230-7.
12 **Russo C**, Angelini G, Dapino D, Piccini A, Piombo G, Schettini G, Chen S, Teller JK, Zaccheo D, Gambetti P, Tabaton M.. Opposite roles of apolipoprotein E in normal brains and in Alzheimer's disease. *Proc Natl Acad Sci USA* 1998;**95**:15598-602.
13 **Wu WS**, Holmans P, Wavrant-DeVrieze F, Shears S, Kehoe P, Crook R, Booth J, Williams N, Perez-Tur J, Roehl K, Fenton I, Chartier-Harlin MC, Lovestone S, Williams J, Hutton M, Hardy J, Owen MJ, Goate A. Genetic studies on chromosome 12 in late-onset Alzheimer disease. *JAMA* 1998;**280**:619-22.
14 **Rogaeva E**, Premkumar S, Song Y, Sorbi S, Brindle N, Paterson A, Duara R, Levesque G, Yu G, Nishimura M, Ikeda M, O'Toole C, Kawarai T, Jorge R, Vilarino D, Bruni AC, Farrer LA, St George-Hyslop PH. Evidence for an Alzheimer disease susceptibility locus on chromosome 12 and for further locus heterogeneity. *JAMA* 1998;**280**:614-18.
15 **Scott WK**, Grubber JM, Conneally PM, Small GW, Hulette CM, Rosenberg CK, Saunders AM, Roses AD, Haines JL, Pericak-Vance MA.. Fine mapping of the chromosome 12 late-onset Alzheimer disease locus: potential genetic and phenotypic heterogeneity. *Am J Hum Genet* 2000;**66**:922-32.
16 **Kang DE**, Saitoh T, Chen X, Xia Y, Masliah E, Hansen LA, Thomas RG, Thal LJ, Katzman R.. Genetic association of the low-density lipoprotein receptor-related protein gene (LRP), an apolipoprotein E receptor, with late-onset Alzheimer's disease. *Neurology* 1997;**49**:56-61.
17 **Lambert JC**, Goumidi L, Vrieze FW, Frigard B, Harris JM, Cummings A, Coates J, Pasquier F, Cottel D, Gaillac M, St Clair D, Mann DM, Hardy J, Lendon CL, Amouyel P, Chartier-Harlin MC. The transcriptional factor LBP-1c/CP2/LSF gene on chromosome 12 is a genetic determinant of Alzheimer's disease. *Hum Mol Genet* 2000;**9**:2275-80.
18 **Taylor AE**, Yip A, Brayne C, Easton D, Evans JG, Xuereb J, Cairns N, Esiri MM, Rubinsztein DC.. Genetic association of an LBP-1c/CP2/LSF gene polymorphism with late onset Alzheimer's disease. *J Med Genet* 2001;**38**:232-3.
19 **Luedecking-Zimmer E**, DeKosky ST, Nebes R, Kamboh MI. Association of the 3'UTR transcription factor LBP-1c.CP2/LSF polymorphism with late-onset alzheimer's disease. *Am J Med Genet* (in press).
20 **Blacker D**, Wilcox MA, Laird NM, Rodes L, Horvath SM, Go RC, Perry R, Watson B Jr, Bassett SS, McInnis MG, Albert MS, Hyman BT, Tanzi RE.. Alpha-2 macroglobulin is genetically associated with Alzheimer disease. *Nat Genet* 1998;**19**:357-60.
21 **Koster MN**, Dermaut B, Cruts M, Houwing-Duistermaat JJ, Roks G, Tol J, Ott A, Hofman A, Munteanu G, Breteler MM, van Duijn CM, Van Broeckhoven C.. The alpha2-macroglobulin gene in AD: a population-based study and meta-analysis. *Neurology* 2000;**55**:678-84.
22 **Luedecking-Zimmer E**, DeKosky ST, Chen Q, Barmada MM and Kamboh MI. Investigation of oxidized LDL-receptor 1 (OLR1) as the candidate gene for Alzheimer's disease on chromosome 12. *Hum Genet* 2002;**111**:443-51.
23 **Iwatsubo T**, Odaka A, Suzuki N, Mizusawa H, Nukina N, Ihara Y. Visualization of A beta 42(43) and A beta 40 in senile plaques with end-specific A beta monoclonals: evidence that an initially deposited species is A beta 42(43). *Neurone* 1994;**13**:45-53.
24 **Hayes A**, Thaker U, Iwatsubo T, Pickering-Brown SM, Mann DM. Pathological relationships between microglial cell activity and tau and amyloid beta protein in patients with Alzheimer's disease. *Neurosci Lett.* 2002;**331**:171-4.
25 **Dignam JD**, Lebovitz RM, Roeder RG. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res* 1983;**11**:1475-89.
26 **Hixson JE**, Vernier DT. Restriction isotyping of human apolipoprotein E by gene amplification and cleavage with Hhal. *J Lipid Res* 1990;**31**:545-8.
27 **MacLean CJ**, Morton NE. Estimation of myriad haplotype frequencies. *Genet Epidemiol* 1985;**2**;263-72.
28 **Yamanaka S**, Zhang XY, Miura K, Kim S, Iwao H. The human gene encoding the lectin-type oxidized LDL receptor (OLR1) is a novel member of the natural killer gene complex with a unique expression profile. *Genomics* 1998;**54**:191-9.
29 **Danik M**, Champagne D, Petit-Turcotte C, Beffert U, Poirier J. Brain lipoprotein metabolism and its relation to neurodegenerative disease. *Crit Rev Neurobiol* 1999;**13**:357-407.
30 **DeMattos RB**, Brendza RP, Heuser JE, Kierson M, Cirrito JR, Fryer J, Sullivan PM, Fagan AM, Han X, Holtzman DM. Purification and characterization of astrocyte-secreted apolipoprotein E and J-containing lipoproteins from wild-type and human apoE transgenic mice. *Neurochem Int* 2001;**39**:415-25.
31 **Schippling S**, Kontush A, Arlt S, Buhmann C, Sturenburg HJ, Mann U, Muller-Thomsen T, Beisiegel U. Increased lipoprotein oxidation in Alzheimer's disease. *Free Radic Biol Med* 2000;**28**:351-60.
32 **Tabner BJ**, Turnbull S, El-Agnaf OM, Allsop D. Formation of hydrogen peroxide and hydroxyl radicals from A(beta) and alpha-synuclein as a possible mechanism of cell death in Alzheimer's disease and Parkinson's disease. *Free Radic Biol Med* 2002;**32**:1076-83.
33 **Keller JN**, Hanni KB, Markesbery WR. Oxidized low-density lipoprotein induces neuronal death: implications for calcium, reactive oxygen species, and caspases. *J Neurochem* 1999;**72**:2601-9.
34 **Keller JN**, Hanni KB, Kindy MS. Oxidized high-density lipoprotein induces neuron death. *Exp Neurol* 2000;**161**:621-30.
35 **Ladu MJ**, Reardon C, Van Eldik L, Fagan AM, Bu G, Holtzman D, Getz GS. Lipoproteins in the central nervous system. *Ann NY Acad Sci* 2000;**903**:167-75.
36 **Wheal HV**, Chen Y, Mitchell J, Schachner M, Maerz W, Wieland H, Van Rossum D, Kirsch J. Molecular mechanisms that underlie structural and functional changes at the postsynaptic membrane during synaptic plasticity. *Prog Neurobiol* 1998;**55**:611-40.
37 **Goritz C**, Mauch DH, Nagler K, Pfrieger FW. Role of glia-derived cholesterol in synaptogenesis: new revelations in the synapse-glia affair. *J Physiol* 2002;**96**:257-63.
38 **Yao ZX**, Papadopoulos V. Function of beta-amyloid in cholesterol transport: a lead to neurotoxicity. *FASEB J* 2002;**16**:1677-9.
39 **Kehoe P**, Wavrant-De Vrieze F, Crook R, Wu WS, Holmans P, Fenton I, Spurlock G, Norton N, Williams H, Williams N, Lovestone S, Perez-Tur J, Hutton M, Chartier-Harlin MC, Shears S, Roehl K, Booth J, Van Voorst W, Ramic D, Williams J, Goate A, Hardy J, Owen MJ. A full genome scan for late onset Alzheimer's disease. *Hum Mol Genet* 1999;**8**:237-45.
40 **Myers A**, Wavrant De-Vrieze F, Holmans P, Hamshere M, Crook R, Compton D, Marshall H, Meyer D, Shears S, Booth J, Ramic D, Knowles H, Morris JC, Williams N, Norton N, Abraham R, Kehoe P, Williams H, Rudrasingham V, Rice F, Giles P, Tunstall N, Jones L, Lovestone S, Williams J, Owen MJ, Hardy J, Goate A. Full genome screen for Alzheimer disease: stage II analysis. *Am J Med Genet.* 2002;**114**:235-44.
41 **Mayeux R**, Lee JH, Romas SN, Mayo D, Santana V, Williamson J, Ciappa A, Rondon HZ, Estevez P, Lantigua R, Medrano M, Torres M, Stern Y, Tycko B, Knowles JA. Chromosome-12 mapping of late-onset Alzheimer disease among Caribbean Hispanics. *Am J Hum Genet* 2002;**70**:237-43.
42 **Blacker D**, Bertram L, Saunders AJ, Moscarillo TJ, Albert MS, Wiener H, Perry RT, Collins JS, Harrell LE, Go RC, Mahoney A, Beaty T, Fallin MD, Avramopoulos D, Chase GA, Folstein MF, McInnis MG, Bassett SS, Doheny KJ, Pugh EW, Tanzi RE. Results of a high-resolution genome screen of 437 Alzheimer's disease families. *Hum Mol Genet* 2003;**12**:23-32.