

Functional interaction between APOE4 and LDL receptor isoforms in Alzheimer's disease

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J Med Genet 2005;42:129–131. doi: 10.1136/jmg.2004.024968

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Received 6 July 2004
Accepted
19 September 2004

Background: Multiple genes have been provisionally associated with Alzheimer's disease, including the coding polymorphisms in exons 8 and 13 in the low density lipoprotein receptor gene (LDLR), situated on chromosome 19p13.2.

Methods: The sample groups consisted of 180 AD patients and 141 control spouses. We carried out genotyping of LDLR8 and LDLR13.

Results: The LDLR8 GG genotype was common, found in 84% of the unaffected control subjects and 91% of the AD patients in our study. There was a ninefold elevation in risk associated with GG:CC versus A– and T– among APOE4+ subjects when compared with APOE4– subjects (odds ratio 9.3; 95% confidence interval 1.8 to 48.2). With the additional information on LDLR polymorphism, we defined an overall 12 fold elevation in risk for APOE4 in combination with LDLR GG:CC (11.9; 2.8 to 50.0; Fisher's exact test, $p=0.0002$; standard power 0.999), compared with other subjects lacking all three of these polymorphisms.

Conclusion: These results imply a functional interaction between ApoE and LDL receptor proteins that determines risk for Alzheimer's disease.

Alzheimer's disease (AD) is a common age related progressive neurodegenerative disorder characterised by severe cognitive impairment. The most prevalent identified risk factor for the disease is the E4 allele of apolipoprotein E (APOE) which accounts for a third or more of the risk when considered as a single factor.^{1–3} ApoE binds the A β peptides that are found in plaques, one of the neuropathological features of an Alzheimer's brain. ApoE4 preferentially binds to the very low density lipoprotein in a complex with cholesterol that binds to the LDL receptor, allowing internalisation of cholesterol into glia.⁴ Binding affinities and efficiencies differ according to isoform (APOE4>APOE3>APOE2).⁵ The LDL receptor belongs to a large family of endocytic receptors, which are found on the neuronal surface and which include LDL receptor related protein and very low density lipoprotein (VLDL). All members of this family bind ApoE. It is thought that ApoE may modulate neuronal plasticity by promoting (ApoE2, ApoE3) or inhibiting (ApoE4) neurite outgrowth.⁶ The LDL receptor gene (LDLR) is located on chromosome 19 (19p13.2), is 45 kb long, and has 18 exons. LDLR has seven functional domains: the promoter translation signal sequence (exon 1), the ligand binding domain (exons 2–6), the epidermal growth factor precursor homology domain (exons 7–14), the O linked sugar domain (exon 15), the membrane spanning domain (exons 16–17), and the cytoplasmic domain (exons 17–18).⁷ More than 600 mutations that are responsible for familial hypercholesterolaemia have been identified in LDLR.⁸

Of relevance to this study, two polymorphisms in exons 8 and 13 in the internalisation EGF precursor homology domain of LDLR have been associated with AD.⁹ The exon 8 polymorphism results in a loss of a *StuI* restriction site; it involves an alanine/threonine (G→A) change at amino acid 370.^{10 11} A relative risk for AD of approximately 2 has been associated with this substitution (1.7 for patients and 2.7 for APOE4 carriers).⁹ The exon 13 polymorphism, introducing an *AvaII* restriction site, involves a T→C substitution at position 632, with no amino acid change.¹² This polymorphism (CC)

has been associated with increased total cholesterol, independent of APOE isoforms.¹² We have investigated these ligand receptor combinations of polymorphisms (LDLR8, LDLR13, and APOE4) in association with the disease state. There was a ninefold increased risk for the LDLR8 GG/LDLR13 CC combination among APOE4 carriers, but no increase in risk for other combinations.

METHODS

Subjects

The sample groups consisted of 180 AD patients and 141 control spouses. The clinical diagnosis of probable AD was made according to NINCDS-ADRDA criteria,¹⁷ following a review of the medical records to verify a documented progressive decline in cognition and appropriate blood tests to rule out other medical conditions, including thyroid and vitamin B12 deficiencies. We also included computed tomography and/or magnetic resonance imaging of the brain, which showed cortical atrophy but no evidence of strokes or tumours. The patients were primarily of European descent. As a group, spouses of patients and siblings had a similar age, ethnic background, and environment, which controlled for unmeasured risk factors in addition to age and race. All participants or those who were the authorised representatives for the patients gave consent for the study, in accordance with institutional review board guidelines.

Patients and control spouses were from both Texas and Georgia. There were 46 Georgian patients (33 women; 13 men) who had a mean (SD) age of onset of 72.04 (8.78) years (range 52–88) and 98 Texan patients (76 women; 22 men); mean (SD) age of onset 70.62 (8.03) years (range 50–92). There were 36 patients (18 women; 18 men) from the National Cell Repository; mean (SD) age of onset 69.58 (7.18) years (range 57–82), making a total of 180 patients (127 women; 53 males); mean (SD) age of onset 70.78 (8.07). Of this total, 144 had an age of onset >65 years. The three

Abbreviations: AD, Alzheimer's disease; APOE, apolipoprotein E; LDLR, low density lipoprotein receptor; VLDL, very low density lipoprotein

groups were analysed separately for population stratification with Structure 2.1 software, and no significant differences were found,¹⁸ thus they were combined for the analysis. There were 141 spouse controls from both patients and siblings (83 women; 58 male) with an average age at ascertainment of 72.27 (8.42) years (range 51–89). Some 121 spouses were >65 years of age at the reference point. The two or three samples that did not amplify were not used in the analysis.

Genotyping

APOE was genotyped as described previously.¹⁹

LDLR8

The primers for the polymorphisms in exon 8 were as described by Retz *et al.*⁹ The conditions for the PCR reactions were 1 μ l 10 \times reaction buffer, 2 μ l “Q”, 0.2 μ l 10 mmol/l each dNTP, 0.4 μ l of 10 μ mol/l each primer, 0.08 μ l of 5 U/ μ l *Taq* polymerase, 100 ng genomic DNA, and H₂O to a final volume of 10 μ l. The PCR conditions for exon 8 were 95°C for 5 minutes, 57°C for 3 minutes, and 72°C for 5 minutes for one cycle, and then 30 cycles of 95°C for 1 minute, 57°C for 1 minute, and 72°C for 1 minute. There was an initial incubation of 95°C for 10 minutes and a final extension of 72°C for 10 minutes. The PCR products were then digested overnight with the restriction enzyme *StuI* (1 μ l of 10 \times buffer, 0.25 μ l of 10 000 U/ml *StuI*, and 3.75 μ l of H₂O plus 5 μ l of PCR product). The DNA fragments were separated by agarose gel (2.0%) electrophoresis and stained with ethidium bromide. The undigested product had a size of 193 bp, and the digested product had sizes of 144 and 49 bp.

LDLR13

The primers for exon 13 were: F: 5'-CAGCCTGGGCAACAAAAGTGAAA and R: 5'-TGGGGCAGAAGAAGCGGAGTC. The PCR conditions were 95°C for 5 minutes, 65°C for 3 minutes, and 72°C for 5 minutes for one cycle, and then 30 cycles of 95°C for 1 minute, 65°C for 1 minute, and 72°C for 1 minute. There was an initial incubation of 95°C for 10 minutes and a final extension of 72°C for 10 minutes. The PCR products were then digested overnight with the restriction enzyme *AvaII*.¹² The DNA fragments were separated by agarose gel (1.5%) electrophoresis and stained with ethidium bromide. The undigested product had a size of 422 bp, and the digested product sizes were 256 bp and 166 bp.

Analysis

Allele/genotype frequencies for case and control subjects were estimated by counting alleles and compared using Fisher's exact or χ^2 tests. Odds ratios (OR) and 95% confidence intervals (CI) were estimated to further quantify risk related to specified combinations of polymorphisms for LDLR8, LDLR13, and APOE. The analysis was implemented in Statistica basic statistics and non-parametric analysis software (version 6.1; StatSoft, Tulsa, OK, USA). Linkage disequilibrium (Arlequin²⁰) and estimated power calculations²¹ were also performed.

RESULTS

LDLR8 GG and LDL13 CC

The LDLR8 GG genotype was common, found for 84% of the unaffected control subjects and 91% of the AD patients (table 1; supplementary tables online). Hence, subjects were divided according to whether or not the LDLR8 GG genotype was present—that is, GG versus A. They were also divided according to whether or not the LDLR13 CC genotype was present—that is, CC versus T, based on the modest elevation of LDLR13 CC among patients (24% *v* 18%) and prior

knowledge that this polymorphism is associated with elevation in plasma cholesterol levels. The component alleles, G and C, were in linkage disequilibrium, (that is, often found together) for the AD patients ($\chi^2 = 15.6$; $p = 0.035$, 4 df), but not for the control subjects ($\chi^2 = 1.1$; $p = 0.89$, 4 df), implying that the double homozygous genotype designated GG:CC may be relevant to disease risk.

APOE4 and LDLR GG:CC

We next considered whether the designated double homozygous GG:CC genotype for LDLR was a risk factor for AD among APOE4+ subjects (table 2). There was a ninefold elevation in risk associated with GG:CC versus A- and T- among APOE4+ subjects (OR 9.3; 95% CI 1.8 to 48.2). This elevation was statistically significant, as the 95% CI did not include the reference value of 1 (that is, no difference in risk for GG:CC versus other possibilities). Moreover, APOE4+ patients carried GG:CC more frequently than did APOE4+ control subjects (Fisher's exact test, $p = 0.008$). These findings indicate that the LDLR polymorphisms strongly modify the risk of AD when APOE4 is present. Specifically, APOE4 in combination with LDLR GG:CC poses high risk.

The LDLR GG:CC genotype did not, however, elevate risk for APOE4- subjects (OR 0.9; 95% CI 0.3 to 2.5). If anything, LDLR GG:CC was mildly protective when APOE4 was not present. Interestingly, subgroup analysis indicated that APOE4+ women who carried the GG:CC combination may be at higher risk compared with men. In addition, those with age of onset >65 years may be more vulnerable. Larger samples are needed to verify these findings.

Overall odds ratio

Some 44% of the patients and 14% of the controls carried at least one APOE4 allele, an approximate threefold elevated risk, on a par with other studies reported in the literature. However, the additional information on LDLR polymorphism defined an overall 12 fold elevation in risk—that is, APOE4 in combination with LDLR GG:CC (OR 11.9; 95% CI 2.8 to 50.0; $p = 0.0002$; standard power 0.999), compared with other subjects lacking all three of these polymorphisms. This further indicates that information on specific LDLR polymorphisms refines information on APOE4 alone when considering the risk for AD.

DISCUSSION

It seems reasonable that particular functional combinations of APOE and receptor isoforms may together modify the risk for AD. The few reports for the LDL family of genes have focused on the oxidised *LDLr1* gene¹³ and on the LDL receptor related protein (*LRP*) gene,¹⁴ both on chromosome 12. Coding polymorphisms for the ligand ApoE and its receptor LDLR, genes that facilitate cholesterol entry into cells, may together serve as risk factors for AD. While there is little evidence as yet of epistasis, there is considerable evidence that genetic variations in APOE and LDLR contribute to the LDL

Table 1 LDLR genotypic frequencies

	Patients	Controls
Exon 8		
GG	161 (90.5%)	116 (84.1%)
AG	17 (9.5%)	21 (15.2%)
AA	0	1 (0.7%)
Exon 13		
TT	55 (30.9%)	38 (27.3%)
TC	80 (44.9%)	76 (54.7%)
CC	43 (24.2%)	25 (18.0%)

Table 2 LDLR GG:CC and risk for AD

	ApoE4+ subjects		ApoE4- subjects	
	Patients	Controls	Patients	Controls
GG:CC	28 (90.3%)	3 (9.7%)	14 (41.2%)	20 (58.8%)
A--:T-	6 (50%)	6 (50%)	11 (44%)	14 (56%)
	OR 0.9; 95% CI 0.3 to 2.5; Fisher's exact test p=0.83; SP 0.06		OR=9.3; 95% CI=1.8 to 48.2; Fisher's exact test p=0.008; SP=0.98	

phenotype.¹⁵ Genetic studies in familial hypercholesterolemia patients have identified over 600 mutations in the *LDLR* gene.⁸ Only two polymorphisms thus far have been associated with AD. In a study of 63 Alzheimer's patients and 162 controls, Retz *et al* found no association of either the exon 8 or the exon 13 polymorphisms in the *LDLR* gene with late onset AD.⁹ They did find that the relative risk for the *LDLR* Thr (exon 8) polymorphism was 1.74 alone and increased to 2.68 when combined with at least one APOE4 allele.

The LDL receptors supply cholesterol to cells and also remove cholesterol rich lipoprotein particles from the bloodstream to prevent their accumulation. The ligand binding domain of the LDL receptor is involved in interactions with lipoproteins containing ApoE. The two polymorphisms in exons 8 and 13 are in the EGF precursor homology domain and are involved with internalisation of the bound ligand with subsequent release in the acidic endosome.⁸ The polymorphisms may prevent the release in the endosome and the subsequent recycling of the receptor. It is interesting to note that APOE4 binds to the LDL receptor with higher affinity than APOE3.⁵ Thus it has been speculated that the receptor may trap APOE4 and reduce its transfer to lipoproteins for clearance, resulting in an increase of cholesterol rich particles in plasma. High levels of cholesterol are thought to increase A β formation. Moreover, elderly people with increased plasma cholesterol levels are at higher risk for dementia.¹⁶ The role of these mutations in the LDL receptor and the interaction with ApoE4 in the brain warrant further investigation.

Thus, we found that there was a ninefold increase in risk for AD for APOE4+ people homozygous for both *LDLR*8 G and *LDLR*13 C. Considered individually, homozygous *LDLR*8 G and homozygous *LDLR*13 each presented a relative risk of about 3 for APOE4+ subjects. Hence, the combination of the two genotypes was highly informative. ApoE4 carriers who carried the double homozygous *LDLR* genotype had a 12 fold increased risk compared with those lacking the components of this tripartite risk factor. These provocative findings need to be investigated in larger samples in conjunction with information on age, sex, race, and clinical features such as cholesterol levels, to potentially become a useful clinical tool.

ACKNOWLEDGEMENTS

We thank the Texas and Georgia families for their active participation in the DNA Bank. DNA was also obtained from the National Cell Repository for Alzheimer's Disease. This research was supported by MCG startup funds, an MCG Dean's summer research fellowship, VA VISN 7 service line career development award, and by a cooperative agreement grant U24AG21886 from the National Institute of Aging for the National Cell Repository.

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Competing interests: there are no competing interests.

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