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ARTICLE

Use of haplotype information to test involvement of the LRP gene in Alzheimer's disease in the French population

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The low density lipoprotein receptor-related protein gene (LRP) is a good candidate gene for Alzheimer's Disease (AD). Its protein is involved in the physiopathology of AD and has been found in senile plaques; on the other hand, LRP is located in 12q, a region in which genetic linkage to AD was reported. Two common polymorphisms, a tetranucleotide repeat in the 5′ untranslated region and a single nucleotide polymorphism at position 766 in exon 3, were found to be associated with AD, but contradictory results were obtained in subsequent association studies. In the absence of clear hypotheses concerning the association of these polymorphisms with AD and their functional role, our objective was to test the association between AD and the two LRP polymorphisms in a large French case-control sample (274 Caucasian AD patients and 290 matched controls) using haplotype analysis. First, the separate study of each polymorphism showed no significant difference in genotype and allele frequencies between AD cases and controls. Second, strong linkage disequilibrium was found between alleles of the two polymorphisms in controls and in cases and the linkage disequilibrium between the 91 bp and C alleles were opposite in cases and in controls. Third, we found that the frequency of the 91-C haplotype was higher in cases than in controls, but the type I error was 0.061, slightly higher than the conventional one of 5%. The haplotype frequencies did not vary significantly as a function of age and APOE ε4 status. One interest in this study is the use of the haplotype analysis, which can be used to combine information from several polymorphisms, taking into account their dependence.
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Introduction

The major known genetic risk factor for non-autosomal dominant Alzheimer's Disease (AD) is undeniably the apolipoprotein E gene (APOE for the gene, apoE for the protein). However, Martinez *et al.*[1] have demonstrated that familial aggregation was not fully explained by this genetic risk factor. Thus, other genes may account for the remaining risk of developing AD. Recently, five linkage

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studies[2–6] reported evidence of an AD susceptibility locus on chromosome 12 where the low density lipoprotein receptor-related protein gene (LRP) is located. LRP is therefore a good candidate gene. Indeed, several observations suggest a potential role for this gene and its protein in the pathogenesis of AD. LRP is a multifunctional ligand receptor expressed in neurons. It is the main apoE receptor in the brain.[7] It mediates neurite outgrowth in an apoE-isoform-dependent manner.[8] It is responsible for the endocytosis of secreted amyloid precursor protein (APP),[9] also implicated in the pathogenesis of AD, and is detected in senile plaques, dystrophic neurites and reactive astrocytes in AD brain.[10]

A genetic association between two different LRP polymorphisms and AD has been reported. The first polymorphism was a (TTTC)n repeat in the 5′ untranslated region (UTR) of the gene. Four different alleles have been described to date, corresponding to 83, 87, 91 and 95 base-pair (bp) PCR products.[11] The 87 and 91 bp alleles are the most frequent. Lendon *et al*.[12] reported a significant increase of the 87 bp allele in AD cases in an American population (P=0.008). In a French population, Lambert *et al*.[13] reported a significant increase in the 91 bp allele in AD cases (P=0.0032), but this association was not confirmed in four other studies.[14–17] The second polymorphism was a silent point substitution in exon 3, in which base 766 is changed from cytosine (C) to thymine (T) but does not modify the amino acid sequence. Kang *et al*.[18] found that the CC genotype was associated with an increased risk for AD (P=0.0024), a finding that was subsequently confirmed by four of six independent studies, including one in France.[16,19–23] Its effect was age-dependent and/or APOE status-dependent in some but not all studies.

Differences in genetic background between American and French populations might explain the contradictory results obtained concerning the first polymorphism. Linkage disequilibrium (LD) between alleles at this locus and a biologically relevant variant elsewhere in the gene may differ among populations. The second polymorphism is a neutral locus. The observed association with AD might only reflect the LD between this polymorphism and the functional variant. It is not therefore on the basis of these polymorphisms that LRP can be considered a genetic risk factor for AD. Another functional variant may be in LD with each of these polymorphisms, which may then be themselves associated with AD by a 'hitchhiking' effect.[24–26] Thomson[24] has shown that a 'hitchhiking' selection event can create LD between neutral loci, which are also passengers in this putative 'hitchhiking' event.

The present study is based on a three-locus 'hitchhiking' model, where one locus is selected, whereas the other two are selectively neutral. We used haplotype information to test the association between AD and the two LRP polymorphisms in a large French case-control sample, taking into account the possible non-independence of the two polymorphisms.

Methods

Subjects

Our sample was composed of 274 unrelated AD cases (44.2% male). All patients were ascertained over a 5-year period through consecutive admissions to several university hospitals in France. The diagnosis of probable AD was established according to the criteria of the National Institute of Neurological and Communicative Disorders and Stroke – Alzheimer Disease and Relative Disorders Association (NINCDS-ADRDA).[27] Age at onset was assessed by interviewing next of kin and was defined as the age at which the patient or his family first noticed the symptoms required for the diagnosis (mean age at onset=65.5 $\pm$ 9.8 years; range=41–90). Patients from families characterised by autosomal dominant inheritance were excluded.

AD patients were compared to 290 age- and sex-matched controls (43.1% male; mean age at examination=67.4 $\pm$ 10.0 years; range=43–93). Control subjects were either patients' spouses, healthy blood donors or individuals living in retirement homes. All controls were screened for cognitive function. None showed cognitive deficit.

All subjects were Caucasian and lived in France. Informed consent was obtained for each subject either directly or from a legal tutor.

Genetic analysis. LRP repeat (TTTC)n

To study the tetranucleotide repeat polymorphism, we have used primers described by Zuliani and Hobbs.[28] PCR amplification was performed in 25 μl of PCR buffer containing 2 mM $MgCl_2$, 7.5 mM dNTP, 1 U of taq DNA polymerase, 15 pmol of each primer and 200 ng of DNA, with 35 cycles of 94°C for 1 min and 60°C for 3 min and a final extension of 7 min at 72°C. Electrophoresis and genotype analyses were performed as described above.

Exon 3 C/T polymorphism

PCR primers were designed to amplify 167 bp around the C/T polymorphism in LRP exon 3 as described by Hollenbach *et al*.[19] The digested products were pooled with the Genscan 500 HD size standard and loaded on a 4.5% denaturing acrylamide gel for electrophoresis with the ABI PRISM 377 DNA sequencer (Perkin Elmer). Each genotype was determined by analysing data with Genscan 3.0 and Genotyper 1.1.1 software.

Statistical analysis

Statistical analyses were performed with the SAS software release 8.0 (SAS Institute Inc, Nashville, TN, USA). For initial comparisons, two-sided χ^2 test and Fisher exact test when necessary were used to determine potential differences in the distribution of LRP genotypes and alleles between groups. For the comparison of each polymorphism separately, the Bonferroni correction was applied for two-group comparisons and the alpha level for each test was set at 0.0253 to bring the alpha level overall back to 0.05. Using this corrected

alpha level, we next estimated the power of the study to detect a difference assuming an OR value of 2, which correspond to an intermediate value between the OR found by Lambert *et al*.[13] (1.6, 95% CI [1.1 – 2.2]) and the one found by Kang *et al*.[18] (2.41, [1.4 – 4.2]). Power computations were made with nQuery Advisor® Release 4.0.

The EH program[29,30] was used to test the existence of linkage disequilibrium between alleles at both loci of the two polymorphisms and to estimate the frequencies of the LRP haplotypes. Haplotype frequencies were estimated in controls and in cases with allelic association (H_1) and without (H_0) by maximising the likelihood, testing first Hardy-Weinberg equilibrium (HWE) in both samples. Departure from HWE may be a substantial source of error in EH haplotype frequency estimations based on the expectation-maximization algorithm, simply because this algorithm uses HWE in its expectation step.[31] The EH program also provides log likelihood, χ^2 and the number of degrees of freedom under hypotheses H_0 and H_1. To compare the homogeneity of haplotype frequencies in cases and controls, a likelihood homogeneity test was performed as follows. Three samples, s_{co} for control, s_{ca} for case and s_p for the pooled sample (cases+controls) were considered. Haplotype frequencies (f_{co}, f_{ca}, f_p) and the corresponding likelihood (L_{co}, L_{ca}, L_p) were estimated by the EH program for controls, cases and pooled sample, respectively. To test the homogeneity of f_{co} and f_{ca}, the quantity $2(\ln L_{co}+\ln L_{ca}-\ln L_p)$ followed a χ^2 test with one degree of freedom. The same principle was applied to test the homogeneity of haplotype frequencies in cases and controls as a function of age (<65 or $\geqslant 65$ years) and APOE $\varepsilon 4$ status (carriers of at least one $\varepsilon 4$ allele or non-carriers).

Results

We first studied the association of each LRP polymorphism with AD. The genotype and allele distributions of these polymorphisms are presented in Table 1. No deviation from the Hardy-Weinberg equilibrium was observed for any of the polymorphisms in controls and AD cases. There was no 95 bp allele in our sample. No significant difference in genotype distributions between AD cases and controls was observed neither for the tetranucleotide polymorphism (P=0.073), nor for the polymorphism in exon 3 (P=0.365). The frequency of the 91bp allele was increased in AD cases compared with controls but the difference was not statistically significant (P=0.231). For the exon 3 polymorphism, allele frequencies were identical between AD cases and controls (P=0.982). Assuming a 0.0253 two-sided significance level, the size of our sample was sufficient to detect a difference with a power of 80% assuming an OR of 2 for developing AD when bearing at least one 91 bp allele[13] and of 84% assuming an OR of 2 when bearing the CC genotype.[18]

Since the two polymorphisms in the LRP gene are physically close to each other, we next tested for linkage disequilibrium between them in controls and cases. Their alleles were in strong linkage disequilibrium (Table 2). Very interestingly, the two at-risk alleles in the French population, the 91 bp and C alleles, were negatively associated in controls and positively associated in cases. The opposite was found for the 91 bp and T alleles. In addition, the 83 bp allele was in complete linkage disequilibrium with the T allele. There was no 83-C haplotype in our sample.

The haplotype frequencies, in cases and controls, estimated by the EH program under the hypothesis of linkage disequilibrium between the alleles of the two polymorphisms are presented in Table 3. The two most frequent haplotypes were 91-C and 87-C. Because the frequencies of the three other haplotypes were lower than 10% in cases and in controls, we decided to pool them. Three haplotypes were therefore compared: two with the at-risk allele C combined with either the 91 bp allele (at-risk in the French population) or the 87 bp allele (at-risk in the American population), and the third consisting of all the other haplotypes pooled. The frequency of the 91-C haplotype was higher in cases than in controls, although the result did not quite reach statistical significance when corrected for the number of haplotypes tested (P=0.061). We have calculated that in our sample, the power to detect an effect of equivalent size (OR=2) to that used to previous power computations was equal to 99%, assuming a 0.05 two-sided significance level when considering an OR for developing AD when bearing the 91-C haplotype. This haplotype was interesting because it contained the two alleles which were found to be at-risk in two other independent French samples. The homogeneity of the frequency of this haplotype was then studied as a function of age and APOE status. Four categories were considered according to age and APOE status (Table 4). Since all these frequencies were estimated by maximisation of likelihood, we estimated their likelihood and then compared them by a likelihood homogeneity test for each category of cases and controls. No differences were significant at 5%.

Discussion

The study of each LRP polymorphism separately showed no significant results after Bonferroni correction. By the haplotypic approach, we found that the 91-C haplotype was more frequent in AD cases than in controls, although statistical significance was borderline at the 5% level when corrected for the number of haplotypes tested. The correction for multiple testing is a major problem in genetic studies in which many polymorphisms are studied without correction. This lack of correction for multiple tests can lead to false-positive results. However, correcting for multiple tests decreases the power to detect true risk factors. One solution is to test an *a priori* hypothesis in a replication study. In the present study, since the 91 bp and C alleles were found to be at-risk in two other French studies with independent samples,[13,21] it would have been possible to test only the increase in the 91-C haplotype versus the others. The

Table 1 Genotype (a) and allele (b) frequencies for each LRP polymorphism

(a) *Genotypes*	*AD cases* (n=*274*)	*Controls* (n=*290*)
(TTTC) n repeat		
83/83	0 (0)	0.3 (1)
83/87	1.1 (3)	1.7 (5)
83/91	2.2 (6)	0.3 (1)
87/87	14.2 (39)	21.0 (61)
87/91	46.4 (127)	42.1 (122)
91/91	36.1 (99)	34.5 (100)
Exon 3		
CC	72.3 (198)	73.8 (214)
CT	25.9 (71)	22.8 (66)
TT	1.8 (5)	3.4 (10)
(b) *Alleles*	*AD cases* (n=*548*)	*Controls* (n=*580*)
(TTTC) n repeat		
83	1.6 (9)	1.4 (8)
87	38.0 (208)	42.9 (249)
91	60.4 (331)	55.7 (323)
Exon 3		
C	85.2 (467)	85.2 (494)
T	14.8 (81)	14.8 (86)

Table 2 Linkage disequilibrium between the alleles of the two LRP polymorphisms in controls (a) and in cases (b). In brackets: maximum possible value of the linkage disequilibrium coefficient, at the given allele frequencies (D' percentage).

(a) *Polymorphism in the 5′ UTR*	*Polymorphism in exon 3* C (%)	T (%)
83 bp	−0.012 (100)	0.012 (100)
87 bp	0.034 (53)	−0.034 (53)
91 bp	−0.022 (34)	0.022 (34)
(b)		
83 bp	−0.014 (100)	0.014 (100)
87 bp	0.012 (21)	−0.012 (21)
91 bp	0.002 (2)	−0.002 (2)

Table 3 Frequencies of LRP haplotypes in AD cases and controls

	AD cases (n=*548*)	*Controls* (n=*580*)
Haplotype		
91-C	51.6 (283)	45.2 (262)
87-C	33.6 (184)	40.0 (232)
Others*	14.8 (81)	14.8 (86)

*91-T, 87-T, 83-T; *P*=0.061.

Table 4 Frequencies of the 91-C haplotype according to age and APOE status

	< 65 APOE4 −	*< 65 APOE4+*	*⩾ 65 APOE4 −*	*⩾ 65 APOE4+*
Cases	57.2 (83)	52.2 (67)	42.3 (46)	54.8 (91)
Controls	42.3 (65)	39.5 (32)	46.2 (139)	61.2 (27)

resulting *P*-value would have been 0.03 and the OR 1.30 (95% CI [1.03 – 1.64]).

It is necessary to apply a correction when two independent tests are performed successively. However, when the two polymorphisms are not independent, as for the two LRP polymorphisms studied, this correction is too conservative. In the extreme case where there is complete linkage disequilibrium between two polymorphisms, testing association with one of the alleles is exactly the same as testing association with the other. This is one strength of the haplotypic approach used in our study. Indeed, haplotype analysis combines information concerning several polymorphisms by taking into account their dependence, rather than studying each polymorphism separately.

The existence of LD between the studied polymorphisms could result in the absence of some haplotypes, as it was the case for the 83-C haplotype in our study. Under the hypothesis that a functional allele is in LD with the polymorphisms studied (three-locus 'hitchhiking' model), LD of opposite signs may be observed in cases and controls, as for the 91 bp and C/T alleles in our study. In this case, the power of a haplotype test to detect the association may be greater than testing the polymorphisms separately (paper in preparation).

As an increasing number of polymorphisms are studied in candidate genes, the haplotypic approach that takes into account the possible non-independence between all these polymorphisms, permits better use of all available information.

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