



## Candidate genes showing no evidence for association or linkage with Alzheimer's disease using family-based methodologies

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### Abstract

Alzheimer's disease (AD) is a genetically complex and heterogeneous disorder. To date, a large number of candidate genes have been associated with the disease, however none of these findings has been consistently replicated in independent datasets. In this study we report the results of family-based analyses for polymorphisms of five such candidates on chromosomes 2 (interleukin-1 $\beta$ , *IL-1B*), 3 (butyrylcholinesterase, *BCHE*), 11 (cathepsin D, *CTSD*; Fe65, *APBB1*) and 12 (lipoprotein receptor-related protein-1, *LRP1*) that were all suggested to be associated with AD in recent case-control studies. To minimize the possibility of spurious findings due to population admixture, we used a family-based design applying the sibship disequilibrium test (SDT) as well as two-point parametric linkage analyses on families from the National Institute of Mental Health (NIMH) Genetics Initiative. Contrary to the initial reports, none of the polymorphisms that were analyzed showed evidence for association or linkage with AD in our families. Our results suggest that the previously reported associations from case-control studies are either (a) false positive results, e.g. due to type I error or population admixture, (b) smaller than initially proposed, or (c) due to linkage disequilibrium with an as yet unidentified polymorphism nearby. © 2000 Elsevier Science Inc. All rights reserved.

**Keywords:** Alzheimer genetics; Linkage studies; Family-based association tests; Candidate genes

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## 1. Introduction

Alzheimer's disease (AD) is a genetically complex and heterogeneous disorder. Although there has been major progress in understanding the genetics of early-onset AD by identifying missense mutations in three genes (*APP*, *PSEN1* and *PSEN2*), the genetic basis of late-onset AD is considerably more difficult to disentangle (Blacker and Tanzi, 1998). To date only one genetic risk factor, a common polymorphism at the apolipoprotein E-locus (*APOE*), has been consistently replicated in independent samples across different ethnic backgrounds (Saunders et al., 1993; Farrer et al., 1997). However, there is considerable evidence that genetic factors other than *APOE* contribute to the risk for late-onset AD: a recent study modelling AD as a quantitative trait estimated at least four additional genetic susceptibility loci for the disease (Daw et al., 2000). One such candidate is the gene encoding  $\alpha$ -2 macroglobulin (*A2M*) on chromosome 12, which is the first putative genetic risk factor for AD that was identified solely by family-based techniques (Blacker et al., 1998).

### 1.1. Strategies to identify novel genes in complex traits

Traditionally, the first step in finding a genetic locus that contributes to a disease is to demonstrate *linkage* with a gene or a DNA sequence of known location. This strategy has led to significant insights in the genetic basis of classic mendelian disorders like Huntington's disease (Gusella et al., 1983), cystic fibrosis (Kerem et al., 1989) or early-onset AD (Blacker and Tanzi, 1998). In complex traits, however, where several genetic loci and/or environmental factors are believed to contribute to disease etiology, linkage studies might not be powerful enough to identify genetic factors of only moderate effect. In this case, linkage of a marker locus to a disease may be suggested by the presence of disease *association* which has been most commonly sought in case-control studies. Despite its relatively good power, the case-control design is prone to spurious findings due to population admixture, where varying allele frequencies within samples, e.g. due to subtle ethnic differences, could give rise to an overall significantly different allele or genotype distribution in cases vs. controls. A robust protection against the bias due to population admixture is afforded by family-based methodologies, where cases and controls are drawn from the same family and, thus, from the same ethnic background (Ott, 1999). In the analysis of putative candidate genes in a complex trait, it therefore becomes increasingly important that a reported association not only be replicated in independent samples of sufficient power, but also that the association be tested using various methodologies (e.g. family-based vs. case-control). Both requirements are fulfilled in the present study, where we analyzed previously associated polymorphisms in five candidate genes in a large sample of multiplex AD families.

### 1.2. Reported candidate genes for AD

Candidate genes were chosen for analysis based on the magnitude of the previously reported association and/or the evidence supporting a possible biological role in AD pathogenesis:

*IL-1B*. Inflammation almost invariably occurs in pathologically vulnerable regions of

AD brains and is thought to exacerbate various other pathogenic processes leading to neurodegeneration (Neuroinflammation Working Group, 2000). Recently, a clinic-based study of more than 600 cases and controls reported an association and significant increase in risk for late-onset AD in carriers of the T/T genotype for a common non-coding polymorphism in the 5' region of the interleukin 1 $\beta$  gene (*IL-1B*, located on chromosome 2p), an immunoregulatory cytokine that is overexpressed in AD brain (Grimaldi et al., 2000). If confirmed, these findings would be the rationale for an early pharmacological control of *IL-1B* mediated neuroinflammation in predisposed individuals that could help slow the progression of neurodegeneration in AD.

**BCHE-K.** Histochemically reactive butyrylcholinesterase (BChE) has been found in the vicinity of amyloid plaques and amyloid angiopathy as well as neurofibrillary tangles in AD brains (Gomez-Ramos et al., 1994). A common polymorphism in the gene encoding BChE (*BCHE-K*, located on chromosome 3q) results in an amino acid change at residue 539 (Ala  $\rightarrow$  Thr) which was found to reduce the catalytic activity of the enzyme by more than 30% (Bartels et al., 1992). A case-control study with 74 pathologically confirmed AD cases and 104 control subjects reported that carriers of the *BCHE-K* variant and at least one *APOE*  $\epsilon$ 4 allele had an almost 7-fold increase in risk of developing late-onset AD as compared to non-carriers (Lehmann et al., 1997). These results are interesting not only because of the reported magnitude of the effect but also because the results were based on pathologically confirmed AD cases.

**CTSD.** Another candidate for genetic association with AD is the gene encoding cathepsin D (*CTSD*), which is located on chromosome 11p. It has been implicated in the processing of amyloid precursor protein (APP) and tau in vitro (Cataldo et al., 1997; Chevallier et al., 1997), i.e. two proteins that are intimately involved in AD neuropathology. A common polymorphism in exon 2 of the gene results in an amino acid change at residue 224 (Ala  $\rightarrow$  Val) which has been associated with increased protein expression (Touitou et al., 1994). Recently, Papassotiropoulos and colleagues reported the results of two independent case-control studies analyzing this polymorphism both of which found a highly significant overrepresentation of the T-allele in AD patients vs. controls (Papassotiropoulos et al., 1999, 2000). From their findings, the authors estimated odds ratios of 2.4 (Papassotiropoulos et al., 1999) and 3.1 (Papassotiropoulos et al., 2000) for developing AD in carriers vs. non-carriers of the T-allele. In addition, carriers of both the T-allele for *CTSD* and at least one  $\epsilon$ 4-allele for *APOE* were reported to be almost 20 times more likely to have AD than non-carriers of these alleles (Papassotiropoulos et al., 2000).

**APBB1.** Approximately 10 cM distal of *CTSD* lies the gene encoding Fe65 (*APBB1*), which binds to the cytoplasmic domain of APP and is thought to be involved in intracellular APP processing (Sabo et al., 1999). Hu et al. identified a polymorphic trinucleotide insertion/deletion in intron 13 of *APBB1*, which interrupts the two exons encoding the APP binding site (Hu et al., 1998). They reported that carriers of the minor allele had a decreased risk for AD vs. non-carriers in a case-control study of 457 subjects (Hu et al., 1998).

**LRPI.** The lipoprotein receptor-related protein (*LRPI*) is the major apolipoprotein E receptor in the brain, and has been shown to mediate endocytosis and degradation of APP (Kounnas et al., 1995; Rebeck et al., 1995). A study of 158 AD cases and 102 controls testing for association of an exonic but silent single nucleotide polymorphism (SNP) in the

LRP1 gene (*LRP1*) was the first to report significant evidence for association of this polymorphism with AD, predominantly in familial forms of the disease (Kang et al., 1997). In the same study, the risk conferring genotype (C/C) was found to not only lower the onset age by approximately 3 years but also to increase the neuritic plaque burden in carriers vs. non-carriers (Kang et al., 1997). The genetic location of *LRP1* falls into a region of chromosome 12 that has been linked to AD in several independent studies (Pericak-Vance et al., 1998; Rogaeva et al., 1998), including analyses performed on the NIMH dataset in our laboratory (Blacker et al., 1998).

## 2. Methods

### 2.1. Patients

Subjects were collected as part of the NIMH Genetics Initiative following a standardized protocol applying NINCDS/ADRDA criteria for the diagnosis of AD (Blacker et al., 1997). This sample presently consists of a total of 1522 subjects in 459 families. The mean age of onset in affecteds was  $71.7 \pm 8.7$  years. In the NIMH study overall, a clinical diagnosis of AD has been confirmed in 94% of the cases that came to autopsy. Approximately half of the sample ( $n = 827$ , affecteds  $n = 460$ , unaffecteds  $n = 367$ ) came from discordant sibships ( $n = 220$ ) and was used in the association analyses, whereas all family members were included in the linkage analyses. To avoid examining very early onset AD, which seems to have a distinct genetic etiology, we included only those families in which all examined affected individuals displayed onset ages greater than 50 years.

### 2.2. Genotyping of *APOE* and candidate genes

*APOE* was genotyped in all subjects as described previously (Blacker et al., 1997). The *IL-1B* -511 C/T SNP was genotyped in 1274 subjects using the same protocol as in the initial study (DiGiovine et al., 1992). The G/A transition at bp 1615 in *BCHE* was genotyped in 860 subjects using the protocol by Jensen et al. (1996). The exon 2 polymorphism of *CTSD* was genotyped in 898 subjects using the same PCR conditions as in the original study followed by an overnight digest with *MwoI* and 6% polyacrylamide gelelectrophoresis (PAGE). The 3 bp insertion/deletion in intron 13 of *APBB1* was amplified by PCR using the protocol described by Hu et al. (1998) and then separated through 6% PAGE in 795 subjects. The exon 3 polymorphism in *LRP1* was genotyped in a total of 817 individuals as reported in the original paper using SSCP analysis on a 5% MDE-gel (BMA, Rockland, ME). All of the above reactions were performed from approximately 30 ng of genomic DNA. Primer oligonucleotides were custom synthesized by Life Technologies (Rockville, MD).

### 2.3. Statistical techniques

To test for association we used the sibship disequilibrium test (SDT) — a family-based association test that does not require parental data (Horvath and Laird, 1998). The SDT is a non-parametric sign test developed for use with sibling pedigree data and compares the

Table 1  
Genotype distribution of the *IL-1B* – 511bp polymorphism in discordant sibships

	Affecteds <sup>a</sup> (n = 377)	Unaffecteds <sup>a</sup> (n = 297)
<i>IL-1B</i> (–511)		
<i>CC</i>	106	83
<i>CT</i>	164	139
<i>TT</i>	107	75

<sup>a</sup> Note that allele- and genotype frequencies are not independent in family-based samples, both within and across affected and unaffected individuals.

average number of candidate alleles between affected and unaffected siblings in each family. Like the TDT and other family-based association tests, this method is not susceptible to bias due to population admixture (Horvath and Laird, 1998). We also performed conditional logistic regression stratified on family, using the individual candidate allele and *APOE*  $\epsilon$ 4-allele carrier status to investigate any effect of these genes separately and together. To test for linkage in the candidate gene region, we performed conventional parametric two-point analyses with FASTLINK (Terwilliger and Ott, 1994) using an autosomal dominant disease model with age-dependent penetrances as described earlier (Blacker et al., 1998). Linkage analyses were performed on the sample as a whole as well as on strata divided by *APOE* genotype.

### 3. Results

Genotype and allele distributions for *IL-1B*, *BCHE* and *LRP1* are displayed in Tables 1–3. The genotypic data for the polymorphisms in *CTSD* and *APBB1* has been reported elsewhere (Bertram et al., 2000; Guénette et al., 2000). Performing the SDT on sibships discordant for AD did not reveal evidence for association in any of the five polymorphisms tested (Table 4). We also did not observe any effect on risk for AD in carriers of the candidate alleles vs. non-carriers, with and without controlling for the presence of *APOE*  $\epsilon$ 4 status in conditional logistic regression (data not shown). Finally, there was no evidence for linkage between AD and any of the polymorphisms tested (maximum LOD scores <1, Table 4). In contrast, we were easily able to detect the effects of the *APOE* polymorphism using the SDT in the NIMH sample: applied to all 827 discordant

Table 2  
Genotype distribution of the *BCHE-K* polymorphism in discordant sibships

	Affecteds <sup>a</sup> (n = 183)	Unaffecteds <sup>a</sup> (n = 154)
<i>BCHE</i> ( <i>K</i> -variant)		
<i>AA</i>	5	4
<i>AG</i>	65	59
<i>GG</i>	113	91

<sup>a</sup> Note that allele and genotype frequencies are not independent in family-based samples, both within and across affected and unaffected individuals.

Table 3  
Genotype distribution of the *LRP1* exon3 polymorphism in discordant sibships

	Affecteds <sup>a</sup> (n = 276)	Unaffecteds <sup>a</sup> (n = 194)
<i>LRP1</i> (exon 3)		
CC	186	135
CT	82	55
TT	8	4

<sup>a</sup> Note that allele- and genotype frequencies are not independent in family-based samples, both within and across affected and unaffected individuals.

siblings both, the overrepresentation of the  $\epsilon 4$ -allele as well as the under-representation of the  $\epsilon 2$ -allele in affecteds vs. unaffecteds were clearly identified ( $p < 1 \times 10^{-7}$  and  $p = 0.00024$ , respectively, data not shown). This suggests that our sample is indeed capable of identifying genetic susceptibility factors of the magnitude of the *APOE* variation — an effect size that was suggested to be met or exceeded in four of the five polymorphisms that were investigated here (*IL-1B*, *BCHE-K*, *CTSD*, *LRP1*).

#### 4. Discussion

Due to the increasing prevalence of AD worldwide it is critical to identify genetic risk factors in parallel with developing therapeutics that can reduce or inhibit the degree of neurodegeneration caused by this devastating disease. Although there is evidence supporting a biological role in AD neuropathology of all five candidate genes that were analyzed in this study (*IL-1B*, *BCHE*, *CTSD*, *APBB1* and *LRP1*), we failed to detect association or linkage between common polymorphisms in these genes and the disease in a large and carefully ascertained family sample. Our findings conflict with recently published studies which detected significant effects for all five variants using a case-control design. As stated in the introduction, it becomes increasingly important in the assessment of candidate genes that not only the initial results be replicated in independent samples of sufficient size, but also by using different analytic approaches to test for association. In AD, only the polymorphisms in *APOE* — and to a more limited extent in *A2M* — meet these requirements, but none of the other polymorphisms reported here has been investigated using a

Table 4  
SDT *p*-values and maximum LOD scores

Polymorphism	Association sample (n)	SDT (p-value)	Linkage sample (n)	Maximum LOD	$\theta$
<i>IL-1B</i> (−511)	674	0.47	1274	0.1	0.3
<i>BCHE</i> (K-variant)	337	0.65	860	0	0.5
<i>CTSD</i> (exon 2) <sup>a</sup>	436	0.68	898	0	0.5
<i>APBB1</i> (intron 13) <sup>b</sup>	526	0.56	795	0.34	0.2
<i>LRP1</i> (exon 3)	470	0.52	817	0.75	0.2

<sup>a</sup> Taken from Bertram et al. (2000).

<sup>b</sup> Taken from Guénette et al. (2000).

family-based methodology after the initial publication. Consistent with our negative results, there have been other independent case-control studies failing to detect significant associations for the polymorphisms in *BCHE* (Brindle et al., 1998; Crawford et al., 1998), *CTSD* (McIlroy et al., 1999) and *LRPI* (Fallin et al., 1997; Woodward et al., 1998). To our knowledge, no published investigation has yet analyzed the association of the *IL-1B* or *APBB1* polymorphisms beyond the initial reports.

There are several possibilities of how our multiple negative findings can be interpreted in the light of the initially reported and highly significant results. As pointed out earlier, all previous investigations were done using case-control analyses and were thus susceptible to spurious findings due to population admixture. Our analyses minimized this bias by employing a family-based strategy. An alternative explanation for our conflicting results is the possibility of a type I error in the initial report. With many different independent laboratories performing a large number of tests to identify new candidate genes worldwide, it is possible that even replicated findings may be due to type I errors, especially in the light of a 'bias' towards publishing positive results in biomedical journals. However, if the associations are genuine our data suggests that they are likely to exert a much smaller effect in the general population than initially proposed, or might be due to linkage disequilibrium with a polymorphism nearby. Both scenarios may be easier to detect in case-control studies rather than family-based analyses due to the inherently lower power of the latter, especially under certain disease models. However, the highly significant results we observed after testing the *APOE* polymorphism individually suggest that our approach using family-based tests on the NIMH families is capable to identify genetic susceptibility factors of the magnitude of the *APOE* variation — an effect size that was suggested to be met or exceeded in four of the five polymorphisms that were investigated here. However, further analyses on even larger samples and more polymorphisms — analyzed individually and jointly — are needed to more clearly elucidate possible effects of the proposed susceptibility genes on the course of neurodegeneration in AD.

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