Association Studies of Transforming Growth Factor-β1 and Alzheimer’s Disease

M.R. Dickson,* R.T. Perry, H. Wiener, and R.C.P. Go
Department of Epidemiology and International Health, University of Alabama at Birmingham, Birmingham, Alabama

Substantial laboratory evidence suggests Transforming Growth Factor-β1 (TGFβ1) is linked to Alzheimer’s Disease (AD) pathology. The purpose of the study was to estimate the genetic association of TGFβ1 with AD while controlling for apolipoprotein E4 allele (APOE4) status, the only well-established genetic risk factor for AD. Two study populations were genotyped for the TGFβ1–509 and +869 single nucleotide polymorphisms (SNPs) that have been associated with TGFβ1 levels. Constituting these populations were 203 families from the NIMH AD Genetic Initiative with at least two affected siblings and a normal sibling, and a population of 126 African-American (AA) AD cases versus 93 age matched controls. Results from family-based analyses showed a significant association between the TGFβ1–509 SNP and AD for the entire set of 203 families (P = 0.007), and a subset of these families without a homozygous APOE4 family member (P = 0.026). Results from family-based analyses on the TGFβ1 +869 SNP were not significant in the 203 families. While associations for the main effects of the TGFβ1 +869 and −509 SNP with AD in the AA case-control study were also not significant, results did indicate that TGFβ1 may function as an effect modifier of APOE4 risk. Specifically, the odds of AD associated with having at least one APOE4 allele increased in an additive fashion with one or two copies of the higher producer allele when stratified by TGFβ1 −509 genotype and by TGFβ1 +869 genotype. Results support a role for TGFβ1 in AD pathogenesis. © 2005 Wiley-Liss, Inc.

KEY WORDS: cytokines; neuroinflammation; apolipoprotein; TGFβ1 +869 SNP; TGFβ1–509 SNP

INTRODUCTION

Alzheimer’s Disease (AD) is a neurodegenerative disorder with a complex etiology and pathogenesis. It is characterized by progressive memory loss and depletion of cognitive functions. The biological processes stimulating the development of AD pathology are not completely understood. One characteristic of neuropathological autopsy findings is the presence of extracellular plaques consisting of aggregates of amyloid-β (Aβ) peptides, which are the proteolytic cleavage products of the amyloid-β precursor protein (APP) [Neeve et al., 2000]. The function of APP is not confirmed, but APP is speculated to support formation and maintenance of synapses in vivo [Mattsson et al., 1997; Wang et al., 2005].

A genetic component of AD has long been recognized, since clustering of this disease within families has been observed [Kamboh, 2004]. The only well-established genetic risk factor for AD is the E4 allele of the apolipoprotein (APOE) gene. The exact role of the APOE protein in AD pathology is not clear, however it is known that APOE functions as a cholesterol transporter, where APOE rich lipoprotein complexes are one source of cholesterol for membrane synthesis and maintenance in the brain [Lahiri et al., 2004; Poirier, 2005]. These complexes also bind Aβ, however, the APOE4 isoform does not form complexes with Aβ peptides, as well as other two APOE isoforms (APOE2 and APOE3), therefore APOE4 may result in lower efficiency in Aβ clearance [LaDu et al., 1997]. Several studies report AD patients homozygous for the E4 allele account for a fraction (<30%) of all AD cases [Kamboh, 2004]. It is well established that APOE4 is only predictive of AD onset and its influence varies across populations [Kamboh, 2004]. Therefore, it remains necessary to consider other genes that might influence the development of disease. Transforming Growth Factor-β1 (TGFβ1) is a multifunctional cytokine with pro- and anti-inflammatory properties and, in particular, is a key regulator of the brain’s responses to injury and inflammation [Mattson et al., 1997; Akiyama et al., 2000]. Within the CNS, TGFβ1 is expressed in neurons, astrocytes, and microglia [Mattson et al., 1997; Harris-White et al., 1998]. Studies show increased TGFβ1 levels in the cerebral spinal fluid of AD patients versus controls, and TGFβ1 is detected in senile plaques [van der Wal et al., 1993; Zetterberg et al., 2004].

Two single nucleotide polymorphisms (SNPs) located in TGFβ1 have been shown to be related to TGFβ1 levels. The T allele of the −509 SNP located in the promoter has been associated with higher plasma concentrations of TGFβ1 in twins, in a dose-dependent fashion [Grainger et al., 1999]. In addition, Luedeking et al. [2000] reported that the T allele was marginally associated with higher transcription activity than the other allele and that the frequency of the TT genotype was significantly higher in AD patients as compared to controls. The +869 SNP is located in the signal peptide region and results in a leucine→proline substitution affecting the protein’s polarity and its export, which could affect protein production [Wood et al., 2000]. With a clinical osteoporosis study and a hypertension study found associations between the +869 C SNP and higher TGFβ1 levels, thus implying that the proline (C) substitution could lead to increased TGFβ1 production [Yamada et al., 1998; Suthanthiran et al., 2000].

In order to investigate the association of TGFβ1 with AD, the −509 C→T promoter SNP and the +869 T→C SNP in the signal peptide region were genotyped in a cohort of 203 families with at least two AD affected siblings and one unaffected sibling from the NIMH AD Genetics Initiative sibling dataset [Blacker et al., 2003] and in an expanded Alabama African-American (AA) case-control cohort (N = 219) [Perry et al., 2005].

Received 28 February 2005; Accepted 2 June 2005

© 2005 Wiley-Liss, Inc.
The purpose of this study is to estimate the effects of TGFBI SNPs on AD risk singly and in the context of APOE4 exposure.

MATERIALS AND METHODS

Study Population

Comprising the family study group were 203 families with at least two AD affected siblings (onset ≥50 years) with mean age of onset (MAO) 71.0 ± 7.5 years and one unaffected sibling. This group (candidate gene set) is a subset of a larger group of families collected as part of the NIMH AD Genetics Initiative [Blacker et al., 2003] following a standardized protocol utilizing the NINCDS-ADRDA criteria for diagnosis of definite and probable AD [Tierney et al., 1988]. Since parent genotypes are not available for this dataset, a subset of families with two affected siblings and at least one unaffected sibling were necessary for family-based statistical analysis. The candidate gene set was further subdivided into 64 APOE4/APOE4 (E4) families with at least one member who is homozygous for the E4 allele (MAO = 76.8 ± 7.1 years), and 139 non-homozygous E4 (NHE4) families where no individuals possess the E4/E4 genotype (MAO = 72.3 ± 7.1 years). Stratifying on homozygous APOE4 status rather than carrier status separates families with strong APOE4 presence from those with no or weak APOE4 presence.

Comprising the AA case-control group were 126 AD cases and 93 age and race matched controls previously reported on by Perry et al. [2001]. AA AD patients had a MAO of 70.8 ± 7.8 years, while the mean age of the control group was 75.2 ± 7.6 years. Cases were identified from patients seen in the Memory Disorder Clinic at the University of Alabama at Birmingham utilizing the NINCDS-ADRDA criteria mentioned above for diagnosis of definite and probable AD. Additional neurological and laboratory tests were completed to exclude cases of other illnesses that can mimic AD. Unaffected spouses of AD patients and members of AD support groups, who screened negative for a personal and family history of memory problems and scored normal on cognitive screening, comprised the control group.

Determination of APOE and TGFBI Genotypes

Blood from the NIMH Genetics Initiative families was collected and sent to the NIMH repository at Rutgers where genomic DNA was extracted from lymphocyte cell lines. Isolation of genomic DNA from the AA subjects has been described [Perry et al., 2001].

APOE4 genotyping followed a modified version of the procedure from Hixson and Vernier [1990] [Blacker et al., 1997]. Primers and enzymes for the PCR-RFLP analysis of the −509 and +869 SNPs of TGFBI were obtained from Luedecking et al. [2000] and Wood et al. [2000], respectively. All PCRs were performed on MJ thermocyclers (Watertown, MA). Amplification of both SNPs were performed in 25 μl reactions containing 0.5 μM of each primer, 1.5 μM MgCl2, 200 μM of each dNTP, 1U Taq (Promega, Madison, WI) and 100 ng of DNA. Conditions were 30 cycles of 30 sec cycling times, with the annealing temperatures for the −509 and +869 SNPs set at 64 and 55 C, respectively. The microtiter plates used in the PCRs were allowed to cool at 12 C/30 min to reduce condensation. Digestions of the −509 and +869 products were performed with 2 and 3U of enzyme (N.E. Biolabs, Beverly, MA), respectively in 15 μl reactions containing 8 μl of product at 37 C/4 hr. Digested products were separated on 2% SFR agarose gels (Amresco, Solon, OH) and photographed on a Fluor-S Imager (Bio-Rad, Hercules, CA). Two independent readers were used for assigning genotypes to reduce errors and clarify any ambiguities.

Statistical Analysis

The family-based association tests implemented in FBAT [Horvath et al., 2001] were used to examine the association of the TGFBI SNPs with AD in the candidate gene family set and the E4 and NHE4 subsets. APOE4 allele dose was controlled for in FBAT in the total candidate gene set by utilizing a difference residual outcome variable from the logistic regression of APOE4 status on AD [Lunetta et al., 2000].

For the AA population, the case and control groups were tested for Hardy–Weinberg equilibrium (HWE) at the TGFBI and APOE loci using the chi-square test. Two sample tests for binomial proportions were used to compare genotype frequencies between cases and controls for each locus. Logistic regression was used to investigate the main effects association of the TGFBI SNPs with AD controlling for APOE4 genotype. Mantel–Haenszel methods were applied to examine the association between APOE4 and AD stratified by TGFBI SNP genotype to further explore the possibility of an interaction between APOE4 and TGFBI that affects disease status [Mantel and Haenszel, 1959].

RESULTS

Results of the family-based association analyses for the −509 (C − T) polymorphism of TGFBI show a significant association (P = 0.007) with AD for the T allele in the candidate gene set, and also marginal evidence for an association in the NHE4 subset (P = 0.026). When controlling for the presence of at least one APOE4 allele in the candidate gene set in FBAT, the association between AD and the −509 SNP remained highly significant (P = 0.007). This association shows the T allele of the −509 (C − T) polymorphism of TGFBI is transmitted more often to the affected siblings. Results from family-based analyses on the TGFBI −869 SNP did not reveal a significant association with AD (data not shown).

The AA case-control population is in HWE for the APOE locus and the TGFBI SNP. Results of baseline comparisons of APOE genotype and the TGFBI genotypes in cases compared to controls show the frequency of the APOE4 genotype differs among cases and controls (P < 0.013) as expected, but the TGFBI genotypes do not (data not shown). The main effects models testing the association of the −509 SNP and +869 SNP genotypes with AD controlling for APOE4 status do not show an association between TGFBI and AD. Analysis of the association of the TGFBI −509 genotype with AD stratified by APOE4 status (having 0, 1, or 2 APOE4 alleles) also showed no significant results, as did the same analysis for the TGFBI +869 SNP (data not shown).

The association of at least one APOE4 allele with AD stratified by TGFBI −509 or +869 SNP genotype is displayed in Table I. Results support the T allele of the −509 SNP and C

<table>
<thead>
<tr>
<th>TGFBI SNP</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>−509 SNP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC (n=115)</td>
<td>2.77</td>
<td>1.28, 6.01</td>
</tr>
<tr>
<td>CT (n=78)</td>
<td>4.02</td>
<td>1.57, 10.33</td>
</tr>
<tr>
<td>TT (n=17)</td>
<td>6.00</td>
<td>0.52, 69.75</td>
</tr>
<tr>
<td>Mantel–Haenszel combined OR</td>
<td>3.35</td>
<td>1.88, 5.98</td>
</tr>
<tr>
<td>+869 SNP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT (n=64)</td>
<td>2.23</td>
<td>0.77, 6.45</td>
</tr>
<tr>
<td>CT (n=92)</td>
<td>3.53</td>
<td>1.49, 8.33</td>
</tr>
<tr>
<td>CC (n=40)</td>
<td>3.43</td>
<td>1.77, 31.04</td>
</tr>
<tr>
<td>Mantel–Haenszel combined OR</td>
<td>3.50</td>
<td>1.93, 6.36</td>
</tr>
</tbody>
</table>

TABLE I. Test for the Association of at Least One APOE4 Allele With AD Stratified by TGFBI −509 or +869 SNP Genotypes in the African-American Case-Control Population
allele of the +869 SNP may modify the effects of APOE4 in an additive manner since the odds ratio between AD occurrence and presence of at least one APOE4 allele increase with each additional copy of the T or C allele. However, these results are only suggestive since the confidence intervals for the odds ratios in the three strata of both SNPs are wide and overlap.

**DISCUSSION**

A significant association between the −509 TGFβ1 SNP and AD was found for the candidate gene set using the family-based test implemented in FBAT ($P = 0.007$). The significant association indicates higher transmission of the −509 T allele to affected siblings as compared to unaffected siblings. When controlling for at least one APOE4 allele in FBAT, the association between the TGFβ1 −509 SNP and AD remained highly significant. The TGFβ1 −509 SNP lies in a negative regulatory element of its promoter, but the T substitution appears to be associated with higher transcription activity and higher plasma levels of TGFβ1 [Grainger et al., 1999; Luedeking et al., 2000]. Our results are similar to a Pittsburgh study that found the TT genotype was significantly higher in AD patients versus controls [Luedeking et al., 2000]. However, a larger group of French sporadic patients as compared to age-matched controls [Ararai-Goumidi et al., 2002] failed to replicate this association.

Like the TGFβ1 −509 T substitution, the +869 C substitution may also affect TGFβ1 production [Wood et al., 2000]; associations of the +869 C substitution with higher TGFβ1 levels have been reported [Yamada et al., 1998; Suthanthiran et al., 2000], however, our results from family-based analyses on the +869 SNP with AD failed to find any association.

For the case-control study in the AA population, there were no associations between the TGFβ1 SNPs and AD. However, the association between carrying at least one APOE4 allele and AD increased when stratified by the TGFβ1 −509 or the +869 SNP genotype. The odds of AD, as determined by APOE4 carrier status, increase in an additive manner based on the number of TGFβ1 −509 T alleles or +869 C alleles (0, 1, or 2) (Table I). Although these differences are not significant due to the wide confidence intervals, it is notable that all six participants who were homozygous for APOE4 and the TGFβ1 +869 C SNP have AD. There are no subjects homozygous for both APOE4 and the TGFβ1 −509 T SNP. Interestingly, these results also show that having no TGFβ1 +869 C alleles dilutes the risk for AD associated with APOE4 carrier status. The above results for the additive effects from the −509 T allele and the +869 C allele in the presence of at least one APOE4 allele lend support for the possibility that TGFβ1 levels may modify the effects of APOE4 protein in AD. Although small sample size restricts the power of these stratified analyses, the consistency of the results between these two SNPs suggests further investigation of a possible synergism between APOE4 and TGFβ1 is warranted.

A model of AD that includes TGFβ1 has biologic support. TGFβ1 increases APP mRNA and protein expression in brain cell cultures [Gray and Patel, 1993; Monning et al., 1994] and appears to increase Aβ deposition in brain cell cultures and in APP and TGFβ1 transgenic mice, when overexpressed [Harris-White et al., 1998; Buckwalter et al., 2002; Lesne et al., 2003]. In addition, one transgenic mouse study showed astroglial overexpression of TGFβ1 produced a strong upmodulation of extracellular matrix (ECM) proteins in the central nervous system (CNS) [Wyss-Coray et al., 1995]. ECM proteins have been identified in Aβ plaques of AD brains where they appear to play a central role in plaque deposition and stabilization. [Fillit and Levey, 1995]. This model of the contribution of T6Fβ1 to plaque formation and stabilization is also consistent with the model in atherosclerosis, another risk factor for AD [Forrester, 2004]. The bulk of severe clinical manifestations of atherosclerosis is attributed to disruption of plaques rich in inflammatory macrophages and T cells, that are characterized by a thin fibrous cap with substantial loss in ECM [Libby, 2001]. Transgenic mice with T cell specific inhibition of TGFβ1 display plaques with these vulnerable properties of plaque T cell infiltration and loss of ECM production [Gjojva et al., 2003]. How does APOE4 fit into this picture? Exposure of cultured neurons to Aβ peptides causes an increase in oxyradical formation and subsequent radical mediated damage to neuron membrane lipids and proteins [Markesbery, 1999]; and the APOE4 isoform has been shown to provide cultured neurons the least antioxidant protection from Aβ generated hydrogen peroxide compared to APOE2 or APOE3 [Miyata and Smith, 1996]. Furthermore, the TGFβ1 promoter contains an element responsive to the transcription factor AP-1 that can be regulated by redox reactions [Iglesias-De La Cruz et al., 2001; Wilm et al., 2002]. If TGFβ1 upregulates APP and Aβ production and deposition, and Aβ induces ROS production, which then upregulates TGFβ1 further, the reduced antioxidant efficiency of APOE4 may lead to exacerbation of the AD pathologic process. Given this model, it is plausible that TGFβ1 may modify the risk for AD associated with the APOE4 isoform by cooperating with Aβ in aggravating the inflammatory process such that it overwhelmed the lesser antioxidant capacity of APOE4.

Our results suggest that TGFβ1 may be associated with AD independently as results from family-based tests indicate or it may function as an effect modifier of APOE4 risk by contributing to a chronic, inflammatory neuropathologic state as results from the case-control association analyses suggest. Biologically both models are feasible, however, both our samples were underpowered to confirm either model. Therefore, we recommend that APOE and TGFβ1 SNPs should be genotyped in family and case/control populations using large sample sizes with sufficient power to distinguish between these two models to confirm our suggestive findings. If either model can be substantiated for TGFβ1 in AD, then the discovery of its exact role in this pathologic process from future cell specific laboratory studies and molecular genetic studies, may provide better insight into the role of cytokines in the neuroinflammatory processes underlying AD pathology, and hence lead to new targets for therapy.

**REFERENCES**


TGFβ1 and Alzheimer’s Disease