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Association of a Haplotype for Tumor Necrosis Factor in Siblings With Late-Onset Alzheimer Disease: The NIMH Alzheimer Disease Genetics Initiative

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Tumor necrosis factor (TNF), a proinflammatory cytokine, may be involved in the pathogenesis of Alzheimer disease (AD) based on observations that senile plaques have been found to upregulate proinflammatory cytokines. Additionally, nonsteroidal anti-inflammatory drugs have been found to delay and prevent the onset of AD. A collaborative genome-wide scan for AD genes in 266 late-onset families implicated a 20 centimorgan region at chromosome 6p21.3 that includes the TNF gene. Three TNF polymorphisms, a –308 TNF promoter polymorphism, whose TNF2 allele is associated with autoimmune inflammatory diseases and strong transcriptional activity, the –238 TNF promoter polymorphism, and the microsatellite TNFa, whose 2 allele is associated with a high TNF secretion, were typed in 145 families consisting of 562 affected and unaffected siblings. These polymorphisms formed a haplotype, 2-1-2, respectively, that was significantly associated with AD ($P = 0.005$) using the sibling disequilibrium test. Singly, the TNFa2 allele was also significantly associated ($P = 0.04$) with AD in these 145 families. This TNF association with AD lends further support for an inflammatory process in the pathogenesis of AD. Am. J. Med. Genet. (Neuropsychiatr. Genet.) 96:823–830, 2000. © 2000 Wiley-Liss, Inc.

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INTRODUCTION

The genetic complexity of Alzheimer disease (AD) and its major expense to society led to the 1990 funding of the National Institutes of Mental Health (NIMH) AD Genetics Initiative. Phase I supported the identification and collection of predominantly late-onset families with AD affected siblings from three sites, the University of Alabama at Birmingham (UAB), Johns Hopkins University (JHU), and Massachusetts General Hospital (MGH). A collaborative genomic screen was performed in Phase II. This work led to the identification of an apparent AD associated deletion in the alpha-2-

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macroglobulin (A2M) gene on chromosome 12 [Blacker et al., 1998] and the detection of a putative AD associated region at chromosome 6p21.3 [Collins et al., 1996; Go et al., 1998].

Within this 6p21.3 region is the major histocompatibility complex (MHC) and human leukocyte antigen (HLA) loci. In 1984, Renvoize reported a weak association between AD and the A2 allele of the HLA-A locus, which was confirmed by Payami et al. [1991]. In 1997, Payami et al. followed this up by reporting that the HLA-A2 allele was associated with a reduced mean age of onset for AD, with a possible additive effect by the apolipoprotein E (APOE) $\varepsilon 4$ allele, which was confirmed by Combarros et al. [1998] and Ballerini et al. [1999].

A candidate gene in this 20 centimorgan (cM) region at 6p21.3 is tumor necrosis factor (TNF, a.k.a. TNFα), which produces a proinflammatory cytokine that helps initiate and regulate cytokine production [Calder, 1997]. TNF increases the production of amyloid β (Aβ) and inhibits the secretion of amyloid precursor protein [Blasko et al., 1999]. However, conflicting results regarding levels of TNF in AD patients have been reported [Alvarez et al., 1996; Lanzrein et al., 1998; Bruunsgaard et al., 1999; Lombardi et al., 1999; Tarkowski et al., 1999]. AD patients have been found to have more TNF receptors than controls, which may indicate systemic immune activation [Bongioanni et al., 1997]. TNF's involvement in inflammation and its effect on Aβ make it an appropriate AD candidate gene.

The TNF –308 and TNF –238 promoter region polymorphisms [Vinasco et al., 1997] and the microsatellite polymorphism TNFa [Martin et al., 1995], located approximately seven kb upstream of TNF, allowed us to test for AD associations in this dataset using family-based association tests. Reported here are the results of the chromosome 6 genomic screen that initially identified the 6p21.3 candidate region and the results of sibling association testing that identified a TNF polymorphism haplotype significantly associated with late-onset AD.

MATERIALS AND METHODS

During Phase I of the NIMH AD Genetics Initiative, 470 AD relative pair families were identified and collected at three clinical sites, UAB, JHU, and MGH. Blood was collected, lymphocytes transformed, and DNA extracted from these cell lines. The Institutional Review Boards of each site approved the human subject research.

In Phase II, UAB typed highly polymorphic microsatellite markers spaced approximately 10 cM apart on chromosomes 1, 6, 14, and 16 (Weber set, ver. 5.0). These and additional flanking markers were used to genotype 266 families that had at least two affected siblings and DNA available. Eighty-four of these families had at least one affected sibling with an APOE $\varepsilon 4/\varepsilon 4$ genotype. The results from the chromosome 6 scan are presented in Figure 1 and Table I.

All microsatellite primers were synthesized in our laboratory (Oligo 1000 DNA synthesizer; Beckman Instruments, Fullerton, CA) or made by Research Genetics (Huntsville, AL). Ten picomoles of the 5′ primer were end-labeled with one microcurie of (γ-^{32}P) ATP (NEN/Dupont, Boston, MA) using one-half unit polynucleotide kinase (Boehringer-Mannheim, Indianapolis, IN) [Maniatis et al., 1989]. Using a 96-well microtiter plate format, PCR was performed in 25 μl reaction volumes containing 100 ng of genomic DNA, 10 picomoles of labeled and nonlabeled primer, and one-half unit of *Taq* polymerase (Promega, Madison, WI). Amplification was performed in an MJ thermocycler (MJ Research, Watertown, MA) at an initial denaturation of 95°C for three min, followed by 35 cycles of 95°C for 40 sec and 55°C for 30 sec. For some primers it was necessary to add DMSO or to adjust the annealing temperature to optimize amplification. After PCR, the products were denatured at 95°C for 3 min and then 2–10 μl of product were size fractionated by denaturing acrylamide gel electrophoresis (6%) followed by autoradiography.

Two independent readers recorded the genotypes and retyped any discrepancies until resolved. The genotypes were entered into the database, LABMAN [Adams, 1994], and checked by two separate individuals. Samples exhibiting mendelization errors or missing typings were repeated. If a sample continued to have mendelization errors it was set blank for that marker. Individuals with mendelization errors over several markers were dropped from the dataset.

One hundred forty-five families with DNA available for at least one affected and one unaffected sibling were

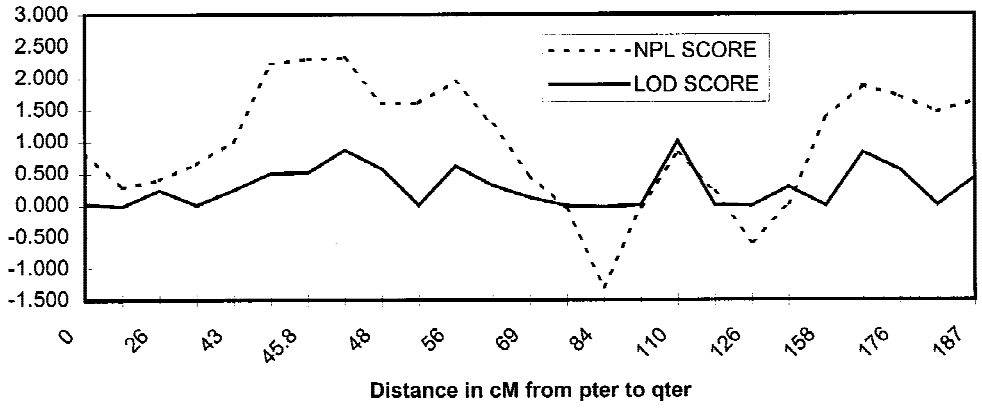

Fig. 1. Graph of the chromosome 6 results for the APOE $\varepsilon 4/\varepsilon 4$ subset.

TABLE I. Results From the 6p21.3 Region in the Total and APOE ε4/ε4 Datasets

UAB Marker	Distance	Stratum (# fams)	SIBPAL Mean	SIBPAL *P*-value	GENEHUNTER NPL score	GENEHUNTER *P*-value	FASTLINK Lod score	FASTLINK Theta
D6S105	43 cM	TOTAL (266)	0.54	0.07	–0.349	0.64	–0.02	0.45
D6S105	43 cM	ε4/ε4 (84)	0.53	0.11	1.012	0.16	0.23	0.20
D6SMIB*	45.7 cM	TOTAL (266)	0.52	0.06	0.777	0.22	0.05	0.35
D6SMIB*	45.7 cM	ε4/ε4 (84)	0.55	0.02	2.225	0.01	0.50	0.15
D6STNFA*	45.8 cM	TOTAL (266)	0.53	0.02	0.886	0.19	0.13	0.30
D6STNFA*	45.8 cM	ε4/ε4 (84)	0.56	0.01	2.301	0.01	0.51	0.15
D6S9N3*	46 cM	TOTAL (266)	0.52	0.06	0.959	0.17	0.06	0.35
D6S9N3*	46 cM	ε4/ε4 (84)	0.56	0.01	2.311	0.01	0.87	0.10
D6S1051	48 cM	TOTAL (266)	0.54	0.02	1.051	0.15	0.16	0.30
D6S1051	48 cM	ε4/ε4 (84)	0.53	0.09	1.610	0.05	0.57	0.10
D6S943	51 cM	TOTAL (266)	0.53	0.14	1.185	0.12	0.04	0.35
D6S943	51 cM	ε4/ε4 (84)	0.50	0.46	1.610	0.05	0.00	0.40
D6S1017*	56 cM	TOTAL (266)	0.52	0.07	1.172	0.12	0.14	0.30
D6S1017*	56 cM	ε4/ε4 (84)	0.55	0.01	1.936	0.03	0.62	0.10
D6S271	65 cM	TOTAL (266)	0.51	0.39	–0.049	0.52	0.01	0.40
D6S271	65 cM	ε4/ε4 (84)	0.53	0.10	1.268	0.10	0.31	0.20

*Indicates flanking marker.

typed for the TNFa microsatellite, the TNF gene polymorphisms at positions -308 and -238 of the promoter region, and the A2 allele of the HLA-A gene. Primers for the TNF promoter polymorphisms and HLA-A2 were purchased from Genosys (The Woodlawns, TX). Genotyping of the TNF promoter polymorphisms followed the protocol of Vinasco et al. [1997], except the α2 and α4 PCR primers required 2.5 mM $MgCl_2$ and an annealing temperature of 55°C. The products were digested with two units of enzyme overnight and electrophoresed on a 3% agarose gel. HLA-A2 was amplified using PCR with sequence-specific primers following the method of the Twelfth International Histocompatibility Workshop [1996]. These products were run on 2% agarose gels.

Nonparametric analyses of marker data from these families were performed using the single-point SIBPAL (S.A.G.E., Case Western Reserve University, Cleveland, OH) and multipoint GENEHUNTER [Kruglyak et al., 1996] programs. A SIBPAL *P*-value of less than 0.05 and a GENEHUNTER NPL score with *P*-value of 0.10 or less was considered a positive finding for follow-up testing using flanking markers. FASTLINK was used to perform parametric maximum likelihood lod score two-point linkage analysis under a dominant model with a 2% gene frequency and 80% penetrance [Cottingham et al., 1993]. A lod score of 1 or above indicated a region for further refinement.

The TNF promoter polymorphisms and TNFa microsatellite were combined to form a haplotype for the TNF region. The most likely parental haplotypes were reconstructed using the GENEHUNTER program [Kruglyak et al., 1996]. Each family's reconstructed haplotypes were then individually inspected to verify their accuracy. If no haplotype assignment could be made for an individual he or she was dropped from the analysis.

After the AD age of onset distributions were evaluated for normality using the Shapiro-Wilk test, mean ages of onset were compared between affected patients with the HLA-A2 allele and affected patients without the HLA-A2 allele using the two-sample *t*-test. This test was also used to compare affected patients that carried the TNF 2-1-2 haplotype to those that did not carry this haplotype. These analyses were performed using SAS Release 6.12 (SAS, Cary, NC).

Association and linkage analyses of AD with the TNF polymorphisms and haplotypes were performed by three family-based association tests that do not require parental genotypes. The SIBASSOC [Curtis, 1997] program performs a χ^2 test using the most genotypically distinct unaffected sibling as a control for each case. This produces positive results only if the marker is associated with and linked to the disease locus, and is similar to the transmission/disequilibrium test (TDT) proposed by Spielman et al. [1993]. The S-TDT [Spielman and Ewens, 1998], which uses marker information from unaffected siblings, was used to test for linkage in sibships containing at least one affected and one unaffected sibling. The final test, the sibship disequilibrium test (SDT), compares alleles of all affected and unaffected siblings in a sibship [Horvath and Laird, 1998] and is a test for linkage as well as linkage disequilibrium.

RESULTS

Initial linkage analyses indicated a 20 cM AD candidate region at 6p21.3 [Collins et al., 1996] in the entire dataset of 266 families (D6S1051 SIBPAL $P = 0.02$; Table I) and the APOE ε4/ε4 subset of 84 families (Fig. 1; D6S1051 GENEHUNTER NPL score $= 1.3$ ($P = 0.10$); multipoint results not shown). Subsequently, flanking markers TNFa and 9N3 both produced GENEHUNTER multipoint NPL scores of 2.3 ($P = 0.01$) and SIBPAL $P = 0.01$ in the APOE ε4/ε4 subset [Go et al., 1998; Table I].

The HLA-A2 phenotype was typed in the 145 families and the ages at onset were squared to achieve normality. Age of onset was not significantly lower ($P = 0.12$) in 164 affected siblings with the HLA-A2 allele (mean = 68.8 years) as compared to 145 affected siblings without the HLA-A2 allele (mean = 70.4 years). The HLA-A2 allele was not found to be associated with AD using family-based association testing.

The TNF promoter polymorphisms were typed in the 145 families (151 sibships) containing 311 affected (69% female; mean age of onset 69.4 years) and 251 unaffected (64% female; mean age at follow-up 72.5 years) siblings. There were no significant differences found when comparing allele frequencies between affected and unaffected siblings (data not shown). Due to the low heterozygosity of these polymorphisms, they were combined with the previously typed microsatellite, TNFa, to create a haplotype in the order of TNF -308, TNF -238, and TNFa. The 2-1-2 haplotype was found to be significantly associated with AD using the SIBASSOC ($P = 0.005$), S-TDT ($P = 0.02$), and SDT ($P = 0.005$) analysis programs. There was also a significant association ($P = 0.04$) between the TNFa 2 allele and AD using the SIBASSOC program. These results are all shown in Table II. Age of onset was not significantly lower ($P = 0.32$) in the 51 affected siblings with the TNF 2-1-2 haplotype (mean = 68.0 years) as compared to the 243 affected siblings without the TNF 2-1-2 haplotype (mean = 69.5 years).

DISCUSSION

The TNF –308 promoter polymorphism TNF2 (G→A) allele, part of the AD-associated haplotype, has been shown to have an increased frequency in autoimmune and inflammatory diseases [Wilson et al., 1995] and is associated with stronger transcriptional activation than the TNF1 allele [Wilson et al., 1997]. The TNFA allele of the –238 TNF promoter polymorphism has no effect on TNF production [Pociot et al., 1995] and our associated haplotype includes the more common –238 TNFG allele. The TNF microsatellite TNFa 2 allele (99 basepairs) has been previously associated with higher TNF secretion [Pociot et al., 1993] and susceptibility to rheumatoid arthritis [Mulcahy et al., 1996, Field et al., 1997].

Thus, two of the TNF alleles comprising this AD haplotype are associated with increased TNF production, which could lead to the chronic inflammatory state and free radical damage hypothesized to be involved in AD pathogenesis [Wood, 1995; McGeer et al., 1996]. This could potentially lead to a lower age of onset for individuals carrying this haplotype. Although the mean age of onset for affecteds carrying the haplotype is 1.5 years lower than the mean age of onset of affecteds not carrying the haplotype, this finding does not reach significance.

TNF has been found in the brain lesions of AD along with other inflammatory cytokines such as interleukin-1 (IL-1), IL-6, and IL-12 [Yen et al., 1995; Fiala et al., 1998]. This chronic inflammatory state could lead to subsequent neuronal damage [Tarkowski et al., 1999] and memory loss [Hauss-Wegrzyniak et al., 1998]. Previous studies have shown that nonsteroidal anti-inflammatory drugs (NSAIDs) protect against or slow the progression of AD [McGeer et al., 1996; Makenzie and Munoz, 1998], with the level of protection directly related to the level of NSAID use [in't Veld et al., 1998]. This protection may be due to the inhibition of cyclo-oxygenase-2 (COX-2), which then reduces the generation of reactive oxygen species harmful to the CNS. COX-2 expression has been shown to be higher in Alzheimer patients [Pasinetti and Aisen, 1998], especially within neurofibrillary tangles [Oka and Takashima, 1997], and TNF has been shown to upregulate COX-2 expression [Geng et al., 1995]. In addition, TNF secretion can be induced by Aβ [Klegeris et al., 1997; Fiala et al., 1998], which upregulates microglia, releasing TNF and free oxygen radicals [Schubert et al., 1998] which can oxidize neuronal proteins [Yatin et al., 1999] and overstimulate the immune system [Behl, 1997; Kaltschmidt et al., 1997]. Therefore, antioxidants may play a key role in protecting the brain from the free radicals [Pitchumoni and Doraiswamy, 1998] produced by Aβ and COX-2 upregulation.

The involvement of free radicals in AD pathology can be linked to the APOE ε4 allele, which is a major risk factor for late-onset AD in its homozygous form [Strittmatter et al., 1993; Farrer et al., 1997; Tang et al., 1998]. The APOE ε4 allele has been shown to have the least antioxidant activity of the three common alleles [Miyata and Smith, 1996]. Therefore, APOE ε3 and ε2 allele protection from free radical damage could explain why AD patients carrying the ε4 allele have lower ages of onset [Corder et al., 1993; Blacker et al., 1997; Meyer et al., 1998]. Furthermore, individuals with dementia have lower levels of the antioxidant vitamins C and E [Riviere et al., 1998; Sinclair et al., 1998]. Vitamin E has also been shown to protect neurons against Aβ toxicity [Behl et al., 1992] and slow the progression of AD [Sano et al., 1997], which further supports the protective role of antioxidants in AD pathogenesis.

The three genes which have been found to cause early-onset AD: the amyloid precursor protein (APP) on chromosome 21 [Goate et al., 1991], presenillin 1 (PS1) on chromosome 14 [Schellenberg et al., 1992], and presenillin 2 (PS2) on chromosome 1 [Levy-Lahad et al., 1995], are proposed to cause AD by increasing the production of Aβ42 [Scheuner et al., 1996; Selkoe, 1996; Citron et al., 1997], which aggregates [Jarrett and Lansbury, 1993] to form neurotoxic AD plaques [Yankner et al., 1989]. Free radicals produced during normal brain metabolism oxidize Aβ and make it aggregate more easily [Dyrks et al., 1992] into this neurotoxic form. We hypothesize that the known early-onset AD mutations upregulate TNF and other cytokines by increasing Aβ production, leading to increased free radical production and senile plaque formation, which eventually leads to neuronal lysis.

A recent study implicating the A2M gene in late-onset AD may also be related to TNF. Blacker et al. [1998] found a deletion in an A2M gene intron that was

TABLE II. Results of the TNF Polymorphism Association Analyses*

Allele	SIBASSOC	S-TDT	SDT
TNF-308 1	$p = 0.55$	$p = 0.08$	$p = 0.17$
TNF-308 2	$p = 0.55$	$p = 0.11$	$p = 0.17$
TNF-238 G	$p = 0.40$	$p = 0.37$	$p = 0.81$
TNF-238 A	$p = 0.40$	$p = 0.91$	$p = 0.81$
TNFa 2	$p = 0.04$	$p = 0.23$	$p = 0.09$
Haplotype 2-1-2	$p = 0.005$	$p = 0.02$	$p = 0.005$

*p-values are not corrected for multiple comparisons.

associated with AD in NIMH families without APOE ε4 alleles, which stayed significant when combined with National Institute of Aging families [Rudrasingham et al., 1999]. Wu et al. [1998] independently found a lod score of 1.91 in AD families without APOE ε4 alleles near the A2M gene on chromosome 12. Additionally, Myllykangas et al. [1999] found an association in exon 24 of the A2M gene in families without APOE ε4 alleles, which was accompanied by an increased level of neuronal Aβ. However, other studies have failed to duplicate this AD association with the A2M gene [Chen et al., 1999; Crawford et al., 1999; Dow et al., 1999; Hu et al., 1999; Rogaeva et al., 1999]. A2M is an acute phase protein and AD plaque component [van Gool et al., 1993; Rebeck et al., 1995] that binds to [Hughes et al., 1998] and degrades Aβ [Qiu et al., 1996]. Additionally, A2M binds TNF [Webb and Gonias, 1998] and may be regulated by the release of TNF and other cytokines [Lyoumi et al., 1998]. This A2M deletion may potentially affect Aβ and TNF binding sites, leading to less degradation, additional plaque formation, and immune stimulation.

AD-affected individuals carrying HLA-A2 in this study did not have a significantly lower mean age of onset than those without HLA-A2. This is not consistent with Payami et al. [1997], but can be explained by the fact that they found a larger difference in sporadic AD patients, while this study consists of familial AD patients. Our AD patients also have a mean age of onset of 69.4 years, while Payami et al. found the most consistent association in early-onset patients. We did not find an association between the HLA-A2 allele and AD, which is consistent with the literature [Payami et al., 1997; Combarros et al., 1998; Ballerini et al., 1999].

Confirmation is still needed to determine if the TNF locus is the primary AD associated gene in this region; however, there is further evidence that this region is implicated in late-onset AD families. Pericak-Vance et al. [1997], in a 54-family late-onset AD genomic screen, found a peak LOD score of 1.37 at marker D6S1019 [Garcia et al., 1999], which maps very close to the TNF gene. Also, Kehoe et al. [1999] found a lod score of 1.4 near the HLA region in a genome screen of 230 families with late-onset AD, which were derived from the same pool of families collected by the NIMH AD Genetics Initiative.

The reconstruction of parental genotypes and haplotypes for these analyses by GENEHUNTER may introduce bias by increasing the type one error rate, especially in families of particular heterozygous parental mating types [Curtis, 1997; Clayton, 1999; Knapp, 1999]. This procedure may also introduce bias by restricting the analysis to families for which a haplotype assignment can be made [Clayton, 1999]. In individual TNF marker analyses using S-TDT and SDT parental genotypes were not reconstructed, but the use of these programs for haplotype analysis may introduce bias, as haplotypes were constructed from sibship genotypes. However, the results from the SIBASSOC test are valid and do not incur the false-positive bias when conditioning on reconstructed haplotypes [Curtis, 1997]. Our dataset consists of 151 sibships, a mean sibship size of 3.7, and a median sibship size of 3, which increases the power of the S-TDT and SDT, and keeps the true type one error rate close to the expected [Knapp, 1999]. Therefore, the increased average sibship size and typing of unaffected siblings allows more accurate reconstruction of parental genotypes [Curtis, 1997; Knapp, 1999] and haplotypes [Clayton, 1999]. Furthermore, only 5% of the siblings (11 affected and 17 unaffected) for whom no haplotype could be assigned were dropped from the analysis.

In this study we chose to examine a broad region associated with AD because it has been established that peaks harboring disease genes are longer than false-positive peaks [Terwilliger et al., 1997], even though the individual screening markers may not meet the stringent criteria discussed by Lander and Kruglyak [1995]. It has been estimated that four additional loci may play a role in late-onset AD [Warwick et al., 2000]; therefore, individual gene contributions may be difficult to elucidate. We realize that with the use of subsets as well as nonparametric, parametric, and association analyses the level of significance of our results may be questioned. However, it should be noted that TNF was the only candidate gene tested in this region. Due to the implication of this region by others, the hypothesized role of TNF in AD, and the complexity of AD genetics, these results merit reporting.

In conclusion, we found that the TNF haplotype 2-1-2, whose alleles are associated with inflammatory diseases and heightened TNF levels, was significantly associated with AD. This, along with the evidence that TNF levels are affected by other known AD mutations and that increased TNF production can lead to an exacerbation of the inflammatory state and free radical generation allows us to hypothesize that increased TNF production can lead to an increased severity of symptoms or decreased onset age in AD patients, for which NSAIDs and antioxidants could be protective. Thus, our results implicating a TNF haplotype lend further support for the possible role of inflammatory cytokines and free radicals in the pathogenic process of AD.

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