Alpha-2 macroglobulin is genetically associated with Alzheimer disease

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Alpha-2-macroglobulin (α-$_2$M; encoded by the gene *A2M*) is a serum pan-protease inhibitor that has been implicated in Alzheimer disease (AD) based on its ability to mediate the clearance and degradation of Aβ, the major component of β-amyloid deposits. Analysis of a deletion in the *A2M* gene at the 5' splice site of 'exon II' of the bait region (exon 18) revealed that inheritance of the deletion (*A2M-2*) confers increased risk for AD (Mantel-Haenzel odds ratio = 3.56, *P* = 0.001). The sibship disequilibrium test (SDT) also revealed a significant association between *A2M* and AD (*P* = 0.00009). These values were comparable to those obtained for the *APOE-ε4* allele in the same sample, but in contrast to *APOE-ε4*, *A2M-2* did not affect age of onset. The observed association of *A2M* with AD did not appear to account for the previously published linkage of AD to chromosome 12, which we were unable to confirm in this sample. *A2M*, *LRP1* (encoding the α-$_2$M receptor) and the genes for two other LRP ligands, *APOE* and *APP* (encoding the amyloid β-protein precursor), have now all been genetically linked to AD, suggesting that these proteins may participate in a common neuropathogenic pathway leading to AD.

AD is a genetically heterogeneous neurodegenerative disorder characterized by global cognitive decline and distinct neuropathological hallmarks in the brain. Defects in three different genes, *APP*, *PSEN1* and *PSEN2*, account for 30–40% of early-onset familial AD (ref. 1). In contrast, late-onset AD (LOAD) has been associated with the risk factor *APOE-ε4* on chromosome 19 (refs 2–4) and *LRP1*, a gene encoding a neuronal receptor for both apoE and APP, the low-density lipoprotein receptor-related protein[5,6]. Family, twin and population data all suggest that other LOAD genes remain to be identified[1].

To identify novel AD genes, we employed a candidate gene strategy focusing on genes for other known LRP ligands. α-$_2$M is a major LRP ligand and abundant serum pan-protease inhibitor[7,8]. In brain, α-$_2$M is upregulated during injury along with LRP (ref. 9) and has been localized to senile plaques (SP) in AD (ref. 10). α-$_2$M binds tightly to Aβ peptide[11,12], the major component of β-amyloid, and attenuates fibrillogenesis and neurotoxicity of Aβ (refs 12,13). α-$_2$M also mediates Aβ degradation[14] and clearance *via* endocytosis through LRP (ref. 15). In view of these findings, we tested for genetic association between *A2M* on chromosome 12p and AD in the National Institute of Mental Health (NIMH) Genetics Initiative AD sample, a large sample of affected sibpairs and other small families with AD (ref. 3). During the course of this study, chromosome 12 linkage to AD was reported as part of a genome screen[16]. As *A2M* maps within 30 cM of the implicated chromosome 12 markers, we also attempted to confirm this result in our sample.

Table 1 • Conditional logistic regression for the effect of *A2M-2* and *APOE-ε4* on risk for AD

Model	Variables	Estimated odds ratio	95% CI	*P*-value
1	*A2M-2* carrier	3.56	(1.80, 7.03)	0.0003
2	*APOE-ε4/ε4*	3.54	(1.76, 7.12)	0.0004
3	*A2M-2* carrier	3.45	(1.71, 6.94)	0.0005
	APOE-ε4/ε4	3.45	(1.67, 7.10)	0.0008
4	*A2M-2* carrier	3.40	(1.57, 7.35)	0.0018
	APOE-ε4/ε4	3.39	(1.51, 7.64)	0.0032
	interaction	1.07	(0.25, 4.46)	0.932

We initially determined genotype counts and allele frequencies for the *A2M* exon 18 splice acceptor deletion[17] (*A2M-2*) in probands, oldest unaffected individuals in each family and 'stringent' unaffecteds (who were at or above the age at which the member of their family with the latest onset of AD was affected; oldest in each family), stratified on individual *APOE-ε4* 'dose'. The combined genotype frequencies for the possession of one or two *A2M-2* alleles were higher in the probands (29.5%) than in the oldest unaffecteds (22.9%) and stringent unaffecteds (19.3%). The *A2M-2* allele frequency for probands (16.4%) was higher than those observed in unaffected individuals (oldest unaffecteds, 12.9%; stringent unaffecteds, 10.5%). The effect was greatest among the *APOE-ε4* zero-dose individuals (probands, 15.0%; oldest unaffecteds, 4.3%; stringent unaffecteds, 3.7%).

Based on all siblings in the 104 families with at least one affected and one unaffected sibling with *A2M* data available, the Mantel-Haenzel odds ratio (Table 1) for being affected as a function of carrying at least one *A2M-2* allele was 3.56 (95% CI = {1.80, 7.03}; *P* = 0.0003). The analogous odds ratio for possessing two copies of *APOE-ε4* was similar in this sample (3.54; 95% CI = {1.76, 7.12}; *P* = 0.0004). Conditional logistic regression analyses adjusted for the effect of *APOE-ε4* on risk for AD are shown (Table 1). Comparable and significant magnitudes of risk were conferred by carrying one or more *A2M-2* alleles (model 1) or by *APOE-ε4* homozygosity (model 2). The magnitude of risk changed little when both genes were included (model 3). There was also no evidence of an interaction between the two genes with regard to conferred risk (model 4). We obtained similar results when we restricted the analyses to stringent unaffecteds.

SDT analysis, a family-based association test that does not require parental data (Horvath, S. and Laird, N., manuscript submitted), revealed an association between *A2M-2* and AD in the total sample (Z = 4.74; *P* = 0.00009) that was comparable to the

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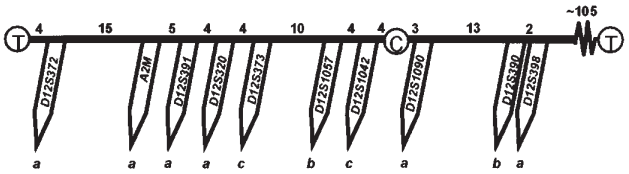

Fig. 1 Map of chromosome 12 markers. The encircled T denotes the telomere; the encircled C denotes the centromere. Map distances are given in centiMorgans (cM). ***a***, Markers used only in the present analysis; ***b***, markers used only in Pericak-Vance *et al.*[16]; ***c***, markers used in both analyses.

magnitude observed for *APOE-ε4* (Z = 4.49; *P* = 0.00006). The *A2M* association persisted in the subset of sibships concordant for zero or one 'dose' of *APOE-ε4*, suggesting that it does not depend on *APOE* status. The effect also persisted when only the stringent unaffecteds were included. Another family-based association test, the sibling TDT (Sib-TDT; ref. 18) also revealed significant association between *A2M* and AD (Z = 3.61, *P* = 0.0002).

Kaplan Meier curves stratified on *A2M* genotype and APOE-4 dose showed that *A2M* genotype had little effect on age of onset irrespective of *APOE-ε4* dose (data not shown). Multivariate analysis of variance of probands also confirmed that there was no effect of *A2M* genotype on age of onset, but did show a significant effect of the *APOE-ε4/ε4* genotype. Thus, whereas *A2M-2* and *APOE-ε4* appear to confer a similar degree of risk for AD, only the dose of *APOE-ε4* lowered the age of onset.

Next, genetic linkage analysis was performed in 286 families for *A2M* and a set of chromosome 12 markers (Fig. 1), including several reported to be linked to AD (ref. 16). *A2M* resides approximately 27 cM from the marker *D12S1042*, which yielded the highest maximum two-point lod score (2.7) by affecteds-only analysis in the previous study[16,19]. Affecteds-only analyses yielded negative lod scores for *A2M* and virtually all markers tested, although the closest flanking markers to *A2M* that were tested were located 5–15 cM from the gene. Lod scores were also negative for the subgroup of families containing at least one affected *APOE-ε4* non-carrier (Tier 3), analogous to that for which linkage to AD was reported on chromosome 12 (ref. 16). Lod scores were also negative in families in which all affecteds were *APOE-ε4* carriers with at least one non-homozygote (Tier 2). In families in which all affecteds were *APOE-ε4/ε4* (Tier 1), there was a non-significant signal (1.23 at Θ = 7 cM for *D12S1042*).

Results obtained with several different age-curve models[16] and multipoint analyses performed in GENEHUNTER and ASPEX gave similar results. Exclusion analyses with 'risk' set at 2 and performed in ASPEX yielded negative lod scores throughout the region for the entire sample (lod < –8), and for Tiers 2 and 3 (lod < –3). All analyses were repeated using uncorrected allele frequencies and defining the sample as described[16], with little change in the results. Thus, we were unable to confirm the previous linkage findings using multiple genetic models, family selection criteria and statistical methods. An independent screen of the NIMH Genetics Initiative AD sample[20] also failed to confirm the reported linkage of AD to chromosome 12 (ref. 16). However, evidence was found for linkage of chromosome 12 to AD using other markers[20] (*D12S98* and *D12S358*) that were located much closer to *A2M* than those used in our genetic linkage analyses. The bi-allelic *A2M* polymorphism that we tested may not have been sufficiently informative for use in traditional genetic linkage analyses in a sample of this nature. These findings also demonstrate the power of family-based association methods for the analysis of candidate genes in this type of sample.

We could not assess mode of inheritance for the observed effect of *A2M-2* on AD. It is also unclear whether *A2M* acts alone or in conjunction with other genes to confer increased risk for AD, or whether there is a differential effect of one *versus* two doses of *A2M-2*. Although *A2M-2* and *APOE-ε4* appear similar in the degree of conferred risk for AD and magnitude of association with AD, *A2M-2* does not appear to share the *APOE-ε4* dose effect on age of onset. One genetic model suggested by these findings would agree with the recent observation, in a study of nearly 5,000 elderly individuals, that *APOE* genotype influences 'when', but not 'whether', AD will develop in *a priori* susceptible individuals[21].

α-$_2$M, a serum pan-protease inhibitor, is expressed in brain and upregulated with its receptor, LRP, during acute-phase brain injury. α-$_2$M has also been shown to bind growth factors, cytokines and small polypeptides[7]. Given the ability of α-$_2$M to tightly bind Aβ (refs 11,12) and mediate its clearance *via* endocytosis through LRP (ref. 15), or degrade Aβ when complexed with a serine protease[14], α-$_2$M may normally prevent accumulation and deposition of Aβ in the brain. In support of this, α-$_2$M has also been shown to attenuate Aβ fibril formation and neurotoxicity[12,13].

APOE promoter polymorphisms, which upregulate transcription of *APOE*, have recently been shown to be associated with AD (refs 22,23). Additionally, higher expression of *APOE-ε4* (relative to *APOE-ε3*) has been reported in the brains of *APOE-ε4*-positive AD patients, but not in age- and genotype-matched controls[24]. Furthermore, the absence of apoE in transgenic mice expressing FAD-mutant *APP* attenuates β-amyloid deposition[25]. One interpretation of these findings and the genetic data presented here is that increased levels of apoE (or apoE4) may confer increased risk for AD by interfering with α-$_2$M-mediated clearance and/or degradation of Aβ by competing with α-$_2$M for either Aβ (ref. 2) or LRP (refs 6,9). In support of this possibility, apoE has previously been reported to inhibit α-$_2$M–mediated degradation of Aβ (ref. 26).

The biological consequences of the 5′ splice-site deletion in exon 18 of *A2M* (ref. 17) have not yet been reported. It is known that exon 18 encodes 'exon II' of the bait domain of α-$_2$M, which is used to attract and trap proteases. It is also possible that association of A2M-2 with AD reflects linkage disequilibrium with another mutation in *A2M* or a nearby gene. In summary, *A2M* appears to be associated with AD by predisposing carriers of the exon 18 splice-site deletion to increased risk for AD, but without modifying age of onset. *A2M*, *LRP1* and the genes encoding two other LRP ligands, *APOE* and *APP*, have now all been genetically linked to AD (refs 1–5,27). Thus, it is plausible that all four of these proteins participate in a common pathogenic pathway leading to AD-related neurodegeneration.

Methods

Sample. The ascertainment and assessment of the AD families collected under the NIMH Genetics Initiative have been described[3]. Briefly, participants were evaluated following a standardized protocol[28] to assure that they met NINCDS/ADRDA criteria for AD (ref. 29), and 142 (22.2%) had autopsy confirmation of the diagnosis. There were also a total of 239 unaffected subjects from 131 families (45.6%). The sample used in the present study consisted of 639 individuals affected with AD, from 286 families. The majority of the affected individuals were sibpairs (202 families, 71%), but there were 46 larger sibships (16%) and 38 families with other structures (13%; parent-child, first cousin, extended). The full sample was used in the descriptive statistics for genotype counts and allele frequencies, for the analyses of age of onset in affected individuals and for all of the genetic linkage analyses (except ASPEX, which uses sibships only). However, because the Mantel-Haenzel test, conditional logistic regression and SDT depend on comparisons of closely related affected and unaffected individuals, these were performed on a subsample including all families in which there was at least one affected and at least one unaffected sibling with A2M data available: 104 families with 217 affected and 181 unaffected siblings.

To avoid examining very early onset AD, which appears to have a distinct genetic aetiology[1], we included only those families in which all examined affected individuals experienced the onset of AD at age 50 or older. Although LOAD is conventionally identified based on onset after age 60, we included families with onsets between 50 and 60 because onset in this decade is only partly explained by the known AD genes. Age of onset was determined based on an interview with a knowledgeable informant and review of medical records.

Amplification and genotyping of *A2M*, *APOE* and chromosome 12 markers. *APOE* was genotyped as described[3]. The *A2M* exon 18 splice-acceptor pentanucleotide deletion was manually genotyped as described[17] in a 96-well format on 6% denaturing polyacrylamide gels. The presence of the deletion resulted in an amplicon smaller by 5 bp, referred to as allele 2 (*A2M-2*). Chromosome 12 markers were manually genotyped with primers that were either provided from the Weber 8 set (Research Genetics) or were custom synthesized (BRL).

Statistical techniques. Five groups of statistical analyses were used to explore the relationship between *A2M* and AD in study families. Wherever possible, we controlled for *APOE-ε4* effects by stratification or by including *APOE-ε4* as a covariate in multivariate analyses. Except as otherwise noted, the analyses reported here were performed in the SAS statistical analysis package (SAS Institute, SAS Program Guide, Version 6).

We examined *A2M* genotype counts and allele frequencies in affected and unaffected subjects in study families. Unaffected individuals in AD families are not genetically independent of their affected relatives, of course, and thus would be expected to show higher frequencies of AD-associated alleles compared with the general population. However, given an increased risk of AD with a given allele, its frequency would be expected to be higher among affected individuals than among their unaffected relatives. As these frequencies are pooled across families, they are neither as accurate nor as powerful an indicator of genetic association as the SDT.

A2M genotype counts and allele frequencies for *A2M-2* in the probands were compared with those for unaffected individuals based on the oldest unaffected individual from each of the 105 families in which one or more unaffected subjects with *A2M* data was available. In addition, the analyses were repeated using an unaffected sample that had passed through a majority of the age of risk, the 'stringent' unaffecteds, who were at or above the age at which the member of their family with the latest onset of AD was affected, again selecting the oldest such individual in each family. Because age of onset is correlated in families[30], using onset ages in the subjects' own families is preferable to setting an arbitrary cutoff.

Mantel-Haenzel odds ratios were calculated for the odds of being affected given the possession of at least one *A2M-2* allele. These analyses were performed stratified on family using n-to-m matching, so all members of a sibship could be used and intercorrelations among siblings could be taken into account. Spielman and Ewens[18] have recently suggested the use of a similar analysis to test for linkage. The analyses were performed first using all unaffected siblings, and then only the stringent unaffected siblings.

Conditional logistic regression was used to control the Mantel-Haenzel odds ratio for the effect of *APOE-ε4* on AD risk. Here, the outcome is disease status of each sibling, conditioning on family using an n-to-m matching paradigm and including *APOE-ε4/ε4* homozygosity as a covariate, along with a term for the interaction between *APOE-ε4* and *A2M-2*. Like the Mantel-Haenzel odds ratio, conditional logistic regression is a standard method for analysis of data from matched sets, and can control for clustering of genotypes within families of arbitrary size. These analyses were performed using the PHREG procedure in SAS. These analyses were repeated using only the 'stringent' unaffected (see above) in order to minimize the effect of misclassification of unaffected siblings.

SDT (Horvath, S. and Laird, N., submitted) is a non-parametric sign test developed for use with sibling pedigree data that compares the average number of candidate alleles between affected and unaffected siblings. The SDT is similar to the Sib-TDT, a recently developed test that also does not require parental data[18], but has the advantage of using all data from sibships of an arbitrary size. Like the TDT, S-TDT and other family-based association tests, the SDT offers the advantage of not being susceptible to admixture errors. Another advantage of these methods is that misclassification of affection status (due to the unaffected siblings not having passed through the age of risk) decreases the power of the test, but does not lead to invalid results. The SDT can test both linkage and linkage disequilibrium; it can only detect linkage disequilibrium in the presence of linkage, hence there is no confounding due to admixture. The null hypothesis of the SDT is that $\Theta = fi$ (no linkage) or $\delta = 0$ (no disequilibrium), i.e., H_0: $\delta(\Theta - fi) = 0$. The STD program (for several platforms) and documentation may be found at: ftp://sph70-57.harvard.edu/XDT/.

Because the SDT does not require parental data, and can use all information from sibships of arbitrary size, it is well-suited to the analysis of the NIMH AD data. Before using it to detect novel AD genes, we validated it with the known AD gene *APOE* in our sample. In an examination of 150 sibships with 286 affected and 242 unaffected individuals from our sample (the number of sibships is higher than that for the *A2M* analyses reported here because a greater number of families have been typed for *APOE*), the SDT was able to detect not only the deleterious *APOE-ε4* effect but also the more difficult to detect *APOE-ε2* protective effect[2–4,31] not previously detected in these data[3] (Wilcox *et al.*, unpublished data).

The primary analysis of the association of *A2M-2* with AD examined the probability of passing along this allele as a function of affection status. In order to increase the likelihood of correct classification of unaffected status, the analyses were repeated including only 'stringent' unaffected siblings (see above), a sample of 60 families. In addition, in order to assess whether the effect was similar across different *APOE* genotypes, the analyses were repeated within strata defined by matching affected and unaffected siblings for *APOE-ε4* gene dose. There were 18 *APOE-ε4* zero-dose sibships, 21 *APOE-ε4* one-dose sibships and 11 *APOE-ε4* two-dose sibships.

To see if *A2M* effects appeared to operate *via* changes in age of onset, we examined affected individuals according to *A2M* genotype, stratifying on or controlling for the powerful effect of *APOE-ε4*. First, we examined this graphically using Kaplan Meier curves (data not shown) including all affected and unaffected individuals, first stratifying on *A2M* genotype alone, and then on *A2M-2* carrier status and *APOE-ε4* dose. Second, we used analysis of variance (ANOVA) on probands, including first only *A2M* genotype (defined as any two *versus* none), then only *APOE* genotype (defined as *APOE-ε4* gene dose or *APOE-ε4/ε4 versus* not), then both, and finally both plus an interaction term.

To determine if there was evidence for linkage in the *A2M* region, and in particular whether *A2M* might be related to the recent reports of linkage to the centromeric region of chromosome 12 (ref. 16), we performed a variety of genetic linkage analytic techniques. For these analyses, all families were divided into 'tiers' according to described criteria[16]: Tier 1 (30 families), families in which all affecteds were *APOE-ε4/ε4*; Tier 2 (131 families), families not in Tier 1 in which all affecteds were *APOE-ε4* carriers; and Tier 3 (126 families), families in which at least one affected did not carry an *APOE-ε4* allele. It should be noted that the previous study[16] found evidence for linkage to chromosome 12 markers only for Tier 3. All analyses were run on the entire sample and on each of these three tiers. In addition, because the previous analyses[16] were confined to families with all onsets at 60 or above, and in which AD was evident in at least two generations, the analyses were repeated using the 259 families meeting these criteria (24 in Tier 1, 118 in Tier 2 and 117 in Tier 3).

The first technique used was conventional linkage analysis using two autosomal dominant disease models.The first model was an affecteds-only analysis based on the model used by Pericak-Vance *et al.*[16]: a gene frequency of 0.001 and a phenocopy rate of 0.05. The other main model was an age-curve model assuming a normal distribution of disease onset with a mean of 71.4 and s.d. of 8.7 (these are the observed values in the NIMH sample, and are very similar to those used in the previous study[16]), a disease gene frequency of 0.01 and a fixed phenocopy rate of 0.10 (this value was used for the probable AD cases (70.3% of the subjects) and for the computation of partial penetrances for unaffected subjects; we used a penetrance of 0.05 for the definite AD cases (22.2% of the subjects) and 0.14 for the possible AD cases (7.5% of the subjects)). However, because these analyses were done in part in an attempt to replicate previous findings[16], additional models (all using the normal onset distribution described above) considered consistent with AD genetics and prevalence were also tested, including our best approximation of the age curve model used in the previous study[16], with a disease gene frequency (q) of 0.001 and a phenocopy rate (φ) of 0.05; $q = 0.01$ and $\varphi = 0.05$; $q = 0.02$ and $\varphi = 0.05$; $q = 0.02$ and $\varphi = 0.10$. All lod score analyses were performed in Fastlink (ref. 32).

The second technique was multipoint non-parametric linkage analysis using the program GENEHUNTER (ref. 33), a multipoint non-parametric

linkage program accommodating full pedigrees, and reporting non-parametric lod (NPL) scores. The last technique was the multivariate sibship analysis package ASPEX (affected sibpair exclusion mapping; ftp://lahmed.stanford.edu/pub/aspex/), which is based on allele sharing within sibships. The analyses were performed using the Sib-Phase program, using fixed allele frequencies based on those observed in the data (see below) to estimate IBD probabilities when parental information is missing, and using no dominance variance. In addition, we performed exclusion analysis setting the ASPEX parameter 'risk' (which is roughly equivalent to λ, the recurrence risk in relatives) at 2, a reasonable value for AD based on family data[30].

For all types of analysis, allele frequencies were computed from the data, but rare alleles were adjusted up to a frequency of 0.01 (with a compensatory small decrease in the frequency of the most common alleles) in order to minimize the possibility of a false positive result. All analyses were repeated using the uncorrected frequencies. For the multipoint techniques (ASPEX and GENEHUNTER), maps of the region were constructed[19]. The map we used (Fig. 1) indicates the markers used in this and a previous study[16].

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