# Genetic association of an α2-macroglobulin (Val1000Ile) polymorphism and Alzheimer's disease

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α2-Macroglobulin (A2M) is a proteinase inhibitor found in association with senile plaques (SP) in Alzheimer's disease (AD). A2M has been implicated biochemically in binding and degradation of the amyloid β (Aβ) protein which accumulates in SP. We studied the relationship between Alzheimer's disease and a common A2M polymorphism, Val1000 (GTC)/Ile1000 (ATC), which occurs near the thiolester active site of the molecule. In an initial exploratory data set (90 controls and 171 Alzheimer's disease) we noted an increased frequency of the G/G genotype from 0.07 to 0.12. We therefore tested the hypothesis that the G/G genotype is over-represented in Alzheimer's disease in an additional independent data set: a group of 359 controls and 566 Alzheimer's disease patients. In the hypothesis testing cohort, the G/G genotype increased from 0.07 in controls to 0.12 in Alzheimer's disease ($P < 0.05$, Fisher's exact test). The odds ratio for Alzheimer's disease associated with the G/G genotype was 1.77 (1.16–2.70, $P < 0.01$) and in combination with *APOE4* was 9.68 (95% CI 3.91–24.0, $P < 0.001$). The presence of the G allele was associated with an increase in Aβ burden in a small series. The A2M receptor, A2M-r/LRP, is a multifunctional receptor whose ligands include apolipoprotein E and the amyloid precursor protein. These four proteins have each been genetically linked to Alzheimer's disease, suggesting that they may participate in a common disease pathway.

## INTRODUCTION

α2-Macroglobulin (A2M) is a proteinase inhibitor which inhibits proteinases of all classes by a steric trapping mechanism. The tetrameric A2M structure undergoes a conformational change upon cleavage of a peptide bond in the bait region by a proteinase and this conformational change traps the proteinase. A2M has been implicated in several pathophysiological processes in Alzheimer's disease. A2M immunostains senile plaques (SP) (1–3) and binds amyloid β protein (Aβ) 1–42 with high affinity (apparent $K_d$ < 1.0 nM) (4). Moreover, recent data suggest that a serine proteinase–A2M complex can degrade Aβ (5) and trypsin-activated A2M efficiently degrades Aβ *in vitro* and prevents *in vitro* formation of thioflavin S-positive Aβ fibrils as well as Aβ-induced toxicity of cultured human cortical neuronal cells (6).

After being activated, A2M is cleared by the A2M-r/low density lipoprotein receptor-related protein (A2M-r/LRP). A2M-r/LRP is a multifunctional receptor and interestingly is also the primary neuronal apolipoprotein E (apoE) receptor (7,8). LRP also binds and clears the Kunitz proteinase inhibitor containing isoforms of the amyloid precursor protein (APP) (9). Thus A2M potentially impacts both apoE and APP metabolism in the brain.

Because of these biological links to Alzheimer's disease, we studied the possible association of a previously reported polymorphism in the A2M gene with Alzheimer's disease. Poller *et al.* (10) screened 30 normal and 30 pulmonary disease patients for A2M polymorphisms at or near the active site and reported three A2M polymorphisms. The most common occurred 25 amino acids downstream from the thiolester site, interchanging Val1000 (GTC) and Ile1000 (ATC) (10). (Numbering is based on the cDNA sequence which includes a 24 amino acid signal peptide; this corresponds to Val/Ile976 in the mature protein.) Allele frequencies in their 60 probands were 0.3 (GTC) and 0.7 (ATC). No difference in A2M serum levels was associated with the two alleles. This polymorphism is especially interesting because it has the potential to be biologically relevant to the function of A2M as a proteinase inhibitor because it occurs near the thiolester active site of the molecule.

## RESULTS

Our initial exploratory experiment tested the possibility that the less common form of A2M (the 'G' allele) would be associated with Alzheimer's disease. Because of the potential for error due to multiple hypothesis testing, we planned to use the initial data

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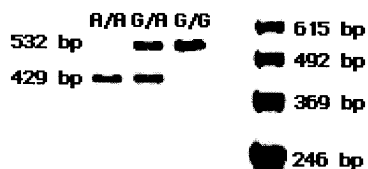
**Figure 1.** Electrophoresis pattern of restriction digest demonstrating bands representing the G (532 bp) and the A alleles (429 bp).

set as an exploratory data set, with the intent to formulate specific hypotheses which would then be tested formally in a second independent data set. We genotyped 90 non-Alzheimer's individuals who had either undergone screening tests with the Blessed dementia scale (11) or whose DNA had been isolated from autopsy material and were demonstrated to not have Alzheimer's disease and 171 individuals who either had a clinical diagnosis of Alzheimer's disease or neuropathologically proven Alzheimer's disease (Table 1). Genotypes were determined by PCR amplification of DNA and restriction enzyme digestion (Fig. 1). In our control series, the G allele frequency was 0.28 and six of 90 individuals contained the G/G genotype (0.067). In the Alzheimer's disease set, the G allele frequency was 0.32. We noticed that the G/G genotype frequency was increased in the Alzheimer's group at 0.12 (Table 1). These results suggest a possible influence of this A2M polymorphism as a genetic risk factor for Alzheimer's disease.

**Table 1.** Over-representation of the A2M G/G genotype in Alzheimer's disease

|  | $n$ | G allele (%) | G/G genotype (%) |
|---|---|---|---|
| Exploratory data set |  |  |  |
| Control | 90 | 0.28 | 0.07 |
| AD | 171 | 0.32 | 0.12 |
| Hypothesis testing data set |  |  |  |
| Control | 359 | 0.32 | 0.07 |
| AD | 566 | 0.34 | 0.12[a] |
| Combined data set (total) |  |  |  |
| Control | 449 | 0.32 | 0.07 |
| AD | 737 | 0.34 | 0.12[b] |

[a]$P < 0.05$; [b]$P < 0.01$.

To formally test the hypothesis that the G/G genotype of A2M is over-represented in Alzheimer's disease, we collected and genotyped additional independent groups of patients and controls. Power analysis showed that >500 Alzheimer's disease and control individuals would be necessary to have an 80% chance of showing a difference between genotype frequencies of 0.07 and 0.12. We therefore collected cases and controls from several sites in order to approximate this number of samples. The second data set consisted of individuals who met the same criteria for Alzheimer's disease or control as the hypothesis generating set and were derived from sporadic Alzheimer's disease patients in Massachusetts, from three European centers and from probands of a multicenter US study of sib pairs and small families. The results from the second data set supported the hypothesis, with G/G genotype frequencies of 0.07 in controls and 0.12 in Alzheimer's disease ($P < 0.05$, Fisher's exact test)

(Table 1). The G allele was not over-represented in this data set (control = 0.32; Alzheimer's disease = 0.34). No difference in age of onset between G/G and non-G/G was found in the second data set. Multivariate analysis showed that site of collection did not influence genotype frequencies. We also compared the rate in controls to the subset of 387 clinic-based Alzheimer's disease cases (G/G frequency = 11.4%, $P = 0.06$) and the subset of 179 familial Alzheimer's disease samples (G/G frequency = 12.7%, $P = 0.056$) and found essentially equal over-representation of G/G in each. These data support an association of the G/G genotype with Alzheimer's disease.

We then carried out a series of exploratory analyses using pooled data from all individuals (737 Alzheimer's disease patients and 449 controls). The G/G genotype was present in 11.9% of the Alzheimer patients and 7.3% of the controls ($P < 0.01$, Fisher's exact test). The genotype frequency in controls was consistent with a Hardy–Weinberg equilibrium. Age of onset was not different between Alzheimer's disease patients with G/G (70.0 ± 9.2, mean ± SD) and non-G/G carriers (70.6 ± 9.1). A multivariate logistic model that controlled for the presence of the *APOE4* allele and gender showed an odds ratio of 1.88 (95% CI 1.20–2.95, $P < 0.01$) for the presence of A2M G/G genotype. The presence of *APOE4* in this model was associated with an odds ratio of 4.21 (95% CI 3.20–5.55, $P < 0.001$). The odds ratio of the combination of G/G and *APOE4* is 9.68 (95% CI 3.91–24.0, $P < 0.001$) relative to those with neither risk factor. To explore whether the G/G-mediated risk was influenced by the presence or absence of *APOE4*, we stratified the sample by the presence or absence of *APOE4*. Similar over-representations of the G/G genotype were seen in the strata with or without *APOE4*, suggesting that the effects of the two risk factors are independent. Multivariate analysis for interaction between the G/G genotype and either *APOE4* or gender demonstrated no interactions ($P > 0.5$ for interaction terms). Analysis based on the Mantel–Haenszel estimator suggested no heterogeneity of odds ratios between strata with and without *APOE4* ($P > 0.5$), consistent with the absence of an interaction between A2M and *APOE4* in our logistic regression model.

We next turned our attention to possible biological effects of inheritance of the A2M G/G genotype. Our previous studies of A2M immunohistochemistry in Alzheimer brain suggested that SP in different cases stained with varying intensity (3). We explored the possibility that this variability was due to different genotypes by immunostaining eight Alzheimer's disease cases known to be G/G or A/A genotype. In all instances, A2M immunoreactivity was present robustly on SP, astrocytes and neurons and no qualitative differences were observed between the genotype groups.

We also explored what effect inheritance of the A2M G/G genotype had on the neuropathological phenotype of Alzheimer's disease (Table 2). We have previously used stereological techniques and quantitative image analysis to measure the amount of Aβ present (amyloid burden) in the neocortical association area surrounding the superior temporal sulcus and the number of neurofibrillary tangles present in the same region (12). Thirty one of these previously analyzed Alzheimer's disease cases were A2M genotyped and selected for further analysis. Initial inspection of the data suggested an increase in Aβ in individuals who were A/G or G/G, with no difference between these two groups. A statistically significant increase in Aβ deposition was seen when comparing the G-containing cases with

the non-G-containing cases ($8.8 \pm 2.8$ versus $6.6 \pm 1.9\%$, $P < 0.03$, unpaired *t*-test). The effect appears to be primarily due to the presence of at least one G allele, in that no difference between A/G and G/G genotypes was observed, although our sample size limits the power of this comparison. There were no statistically significant differences in neurofibrillary tangle number with G alleles, suggesting a specific effect on A$\beta$ deposition.

**Table 2.** Neuropathological correlates of the A2M genotype

| Genotype | $n$ | Amyloid burden (%) | Neurofibrillary tangles ($\times 10^3$) |
|---|---|---|---|
| A/A | 10 | $6.6 \pm 1.9$[a] | $6.9 \pm 4.2$ |
| A/G + G/G | 21 | $8.8 \pm 2.8$[b] | $9.0 \pm 5.0$[c] |

[a]Mean $\pm$ SD.
[b]$P < 0.03$, *t*-test.
[c]$n = 17$ for this measurement.

## DISCUSSION

Exploratory studies of genetic risk factors run the risk of type I errors because of the large number of hypotheses that are either explicitly or implicitly being tested. Alternatively, type II errors can occur if sample size is insufficient. In order to overcome some of the difficulties inherent in exploratory studies of genetic risk factors we used independent data sets to first generate, then test the hypothesis that the G/G genotype is associated with Alzheimer's disease. Two separate control populations gave identical genotype frequencies; our hypothesis testing data set was itself made up of two groups (sporadic AD and probands from a multicenter study of Alzheimer's disease sib pairs and small families) each of which gave essentially identical genotype frequencies and over-representation of the G/G genotype. The A2M G/G effect was not dependent on *APOE* genotype and the effect was additive and substantial: the odds ratio of G/G in the presence of an *APOE4* allele showed an almost 10-fold increased risk. Power analysis using the size of effect observed in our combined data set (using ~500 samples) suggests that a group size of 578 controls and 578 Alzheimer's disease patients would be necessary to have an $\alpha$ of 0.05 and a $\beta$ of 80% for the hypothesis that the G/G genotype is increased from 0.07 to 0.12 in Alzheimer's disease.

The association of the G/G A2M genotype as a risk factor in Alzheimer's disease leads to several interesting mechanistic interpretations. Previous data have shown that A2M immunostains SP and have implicated A2M in amyloid precursor protein metabolism (13) as well as possible interactions with binding of A$\beta$ (4) and mediation of degradation of A$\beta$ (5,6). A2M–A$\beta$ complexes undergo endocytosis via the A2M-r/LRP receptor (14). A2M associates with A$\beta$ and prevents fibril formation (15). In addition, A2M can be viewed as a (sub)acute phase reactive molecule and as a potent proteinase inhibitor which might participate in the inflammatory response in Alzheimer brain. Interestingly, A2M has also been implicated as a trophic factor, possibly as a result of binding and transporting a number of polypeptide growth factors and cytokines (16). Like apoE, A2M may serve as a binding protein for targeting various bioactive molecules to their site of action or clearing them (3). Finally, the fact that apoE and APP, two other ligands of the A2M-r/LRP receptor are clearly involved in the pathophysiology of Alzheimer's disease, support the plausibility of the idea that an A2M polymorphism might contribute to risk of Alzheimer's

disease. In fact, a polymorphism in A2M-r/LRP itself has recently been reported in two studies to show a genetic association with Alzheimer's disease (17,18).

Although there is a strong biological rationale for the involvement of an A2M polymorphism in Alzheimer's disease, the possibility remains that the genetic association we observe reflects linkage with another mutation or polymorphism either in the A2M gene itself or in a nearby gene on chromosome 12. The A2M gene resides ~25 cM distal of the marker D12S1042, which yielded the maximal LOD score in a recent linkage study, suggesting that chromosome 12 contains an Alzheimer risk factor (19). However, the likelihood that A2M itself is a risk factor is supported by the results of a recent separate study, in which we observed an association between an intronic (5′ splice site of exon 18) pentanucleotide deletion (allele 2) within the A2M gene and Alzheimer's disease in a sibship analysis of Alzheimer's disease sib pairs and small Alzheimer's disease families (20). Our current results extend this observation by showing linkage of a missense mutation in a case–control sample rather than family-based association. We are currently studying how the two A2M polymorphisms relate to one another; our preliminary results are consistent with the hypothesis that they act as independent risk factors. Initial study of a group evaluated for both polymorphisms suggests that the G allele is inherited with the 1 allele; of 70 G chromosomes examined (35 G/G individuals), 97% were allele 1 and 3% were allele 2, compared with an allele 2 frequency of ~15% ($P < 0.005$, $\chi^2$ analysis with Yate's correction).

Taken together, we believe that polymorphisms in the A2M gene may contribute to risk for Alzheimer's disease. Moreover, these data focus attention on mechanisms of pathophysiology that impact on A2M-r/LRP or its ligands, including clearance mechanisms mediated by A2M-r/LRP (7,14), and suggest that other A2M-r/LRP ligands may be candidate Alzheimer's disease genes as well.

## MATERIALS AND METHODS

### Subjects

An initial hypothesis generating data set was established from 171 Alzheimer's disease patients with a clinical history of probable Alzheimer's disease and 90 age-compatible control individuals, who were primarily spouses of the patients. A second hypothesis testing data set was collected from several sources in an attempt to approximate the >500 patients and controls estimated by power analysis to adequately test the hypothesis that the G/G genotype is over-represented in Alzheimer's disease. These individuals included additional cases of 380 sporadic Alzheimer disease and 337 controls matched for site (primarily cognitively tested spouses) collected from the Massachusetts General Hospital Memory Disorder Unit and from a consortium of European Centers (University of Hamburg, Germany, University of Basel, Switzerland and S. Cuore Fatebenefratelli Hospital, Brescia, Italy) and 179 probands from a multicenter study of Alzheimer disease sib pairs and small Alzheimer's disease families (21).

### A2M polymorphism genotyping

Genomic DNA isolated from brain tissue and blood was amplified by PCR in the presence of oligonucleotide sense primer C23 (5′-ATC CCT GAA ACT GCC ACC AA-3′) and antisense primer AS24 (5′-GTA ACT GAA ACC TAC TGG AA-3′), 10 mM Tris–HC1, 50 mM KC1 (pH 8.3), 1.5 mM MgC1$_2$, 5 mM

dNTPs, 5 pmol each primer and 1.25 U Taq DNA polymerase. The PCR was carried out in a touchdown procedure that stepped down the annealing temperature to increase primer specificity as follows: one cycle at 94°C for 5 min; four cycles at 94°C for 30 s, 65°C for 30 s, 72°C for 1 min; four cycles at 94°C for 30 s, 62°C for 30 s, 72°C for 1 min; four cycles at 94°C for 30 s, 59°C for 30 s, 72°C for 1 min; 20 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 1 min; one cycle at 72°C for 5 min.

In each reaction mixture, *Mbo*I was added to the amplified product of 615 bp and digestion carried out at 37°C for 3 h, producing fragments of 532 (G allele) or 429 bp (A allele). The digested product was loaded onto a 2% agarose gel treated with ethidium bromide (0.005%) and electrophoresed for 2 h under constant voltage (150 V), which is sufficient to separate the digested product so that the 532 and 429 bp bands can be distinguished. After electrophoresis, DNA fragments were visualized by UV illumination using a Bio-Rad Gel Doc system. Incomplete digestion was monitored by the continued presence of the 615 bp product.

Immunohistochemical analysis used anti-A2M antibody (1:500) from Zymed. A goat Cy3-linked secondary antibody (Jackson Immunoresearch) was used to visualize immunostaining. In some instances, double immunofluorescence was carried out with an Aβ counterstain (antibody 10D5, courtesy of Dr D. Schenk, Athena Neurosciences) using bodipy-fluorescein-linked secondary antibody (Molecular Probes) as the second fluorochrome. Quantitative neuropathological techniques for measuring Aβ burden and neurofibrillary tangles number in the superior temporal sulcus area have been previously published (12).

## Statistical methods

Comparisons of A2M allele frequency (proportion of chromosomes in which an allele is present) and genotype frequency (proportion of individuals with a genotype) were performed with $2 \times 2$ tables using Fisher's exact test for significance. Age of onset of Alzheimer's disease was normally distributed and compared by *t*-test. Multivariate analysis for odds of Alzheimer's disease was performed by logistic regression with *APOE* genotype coded according to the presence or absence of *APOE4*. Similar results were obtained when the group with *APOE4* was coded separately as heterozygotes or homozygotes for this allele. Odds ratios are presented with 95% confidence intervals (CIs). All analyses were performed with Stata software (College Park, TX). All significance tests were two-tailed.

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