Objective: To determine whether the cystatin C gene (CST3) is genetically associated with late-onset Alzheimer disease (AD).

Design: A case-control study with 2 independent study populations of patients with AD and age-matched, cognitively normal control subjects.

Setting: The Alzheimer’s Disease Research Unit at the University Hospital Hamburg-Eppendorf, Hamburg, Germany, for the initial study (n=260). For the independent multicenter study (n=647), an international consortium that included the Massachusetts Alzheimer’s Disease Research Center at the Massachusetts General Hospital, Boston; the Scientific Institute for Research and Patient Care, Brescia, Italy; and Alzheimer’s research units at the Universities of Basel and Zurich, Switzerland, and Bonn, Goettingen, and Hamburg, Germany.

Participants: Five hundred seventeen patients with AD and 390 control subjects.

Measures: Molecular testing of the KspI polymorphisms in the 5’ flanking region and exon 1 of CST3 and the apolipoprotein E (APOE) genotype. Mini–Mental State Examination scores for both patients with AD and control subjects.

Results: Homozygosity for haplotype B of CST3 was significantly associated with late-onset AD in both study populations, with an odds ratio of 3.8 (95% confidence interval, 1.56-9.25) in the combined data set; heterozygosity was not associated with an increased risk. The odds ratios for CST3 B/B increased from 2.6 in those younger than 75 years to 8.8 for those aged 75 years and older. The association of CST3 B/B with AD was independent of APOE e4; both genotypes independently reduced disease-free survival.

Conclusions: CST3 is a susceptibility gene for late-onset AD, especially in patients aged 75 years and older. To our knowledge, CST3 B is the first autosomal recessive risk allele in late-onset AD.
SUBJECTS AND METHODS

SUBJECTS

We conducted molecular genetic studies on 2 independent study populations: an initial sample (n = 260 participants), and a multicenter sample (n = 647 participants). The clinical diagnoses of AD were made according to the NINCDS-ADRDA criteria; 84% of our patients had Mini-Mental State Examination (MMSE) scores lower than 24, similar to previous studies of AD. The control subjects were not demented, and had MMSE scores of 27 or higher. All participants in this study were Caucasian. Informed consent was obtained from all participants, and the local human studies committees approved the study protocol.

The initial sample included 110 patients with AD (aged 73.1 ± 8.9 years [mean ± SD]; 79 women) and 150 nondemented control subjects (aged 65.2 ± 14.6 years [mean ± SD]; 66 women) from the Alzheimer’s Disease Research Unit at the University Hospital Hamburg-Eppendorf, Germany. An independent hypothesis-testing sample was collected according to identical standardized criteria by an international multicenter consortium that included AD research units in the United States, Italy, Germany, and Switzerland. This sample consisted of 407 patients with AD (aged 75.0 ± 9.5 years [mean ± SD]; 284 women) and 240 age-matched nondemented controls (aged 75.1 ± 6.3 years [mean ± SD]; 139 women).

GENOTYPING

Preparations of DNA and polymerase chain reactions (PCRs) were performed following standard protocols. The 318-base pair (bp) PCR product generated with primers 024F (TGGGAGGGACGAGGCGTTCC) and 1206R (TCATGGGCTCCTCCCCACCA) was designed to cover all 3 polymorphic KspI sites described above. Digestion with KspI generated 3 fragments of 41, 226, and 51 bp in size for haplotype A, or 2 fragments of 127 and 191 bp for haplotype B. These banding patterns allowed us to determine the phase of the polymorphisms. Among the 907 samples genotyped in this study, there was no case of aberrant banding pattern in this assay, confirming that no other haplotypes defined by these 3 polymorphic sites were present in our study population. In addition, we confirmed these haplotypes by direct sequencing of PCR products from subjects with the respective genotypes A/A, A/B, or B/B. APOE was routinely genotyped by using a standard PCR- and restriction-based protocol. The observed CST3 and APOE genotype counts did not significantly deviate from those expected under the Hardy Weinberg equilibrium of patients and controls in the initial, the multicenter, or the combined samples.

penultimate position of the signal peptide. Because of linkage disequilibrium, these 3 polymorphisms in the CST3 gene result in only 2 commonly found human haplotypes, called CST3 A (nucleotides G, A, and G at positions −157, −72 and +73) and CST3 B (C, C, and A at these positions), respectively. CST3 A (nucleotides G, A, and G at positions −157, −72 and +73) and CST3 B (C, C, and A at these positions), respectively.

RESULTS

INITIAL STUDY

There was a significant association between AD and CST3 genotype (\( \chi^2 = 6.67; 2\)-sided Fisher exact test, \( P = .04 \)) (Table 1). The borderline higher frequency of allele B (F2) in patients (25%) than in controls (18%) (Pearson \( \chi^2 = 3.75; P = .05 \)) was due to an excess of B/B homozygotes in the patients with AD (9.1%) compared with the control subjects (2.0%). The proportion of A/B heterozygotes in the patient and control groups was almost identical. These data suggested an odds ratio (OR) for AD of 4.9 (95% confidence interval [CI], 1.32-18.25) in association with the homozygous genotype B/B. As expected, there was also a highly significant association between APOE genotype and AD. Of the patients with AD, 13.6% were homozygous for the e4 allele, compared with 2.7% of the control subjects; 50.0% of patients with AD were heterozygous ε3/ε4 or ε2/ε4 (control subjects, 29.3%), and 36.4% of the patients with AD had no e4 allele, vs 68% of the controls (\( \chi^2 = 29.2; P < .001 \)). Nonconditional logistic regression analysis revealed a comparably strong effect of CST3 B/B on the individual risk for AD as the presence of an APOE e4 allele (Table 2). Nonconditional logistic regression analyses revealed significant effects on the risk for AD of APOE e4, CST3 B/B,
sex, and age, but we did not find evidence for significant interactions between APOE and CST3.

**MULTICENTER STUDY**

There were no significant differences in CST3 allele frequencies in the independently collected control and patient samples obtained by the multicenter consortium: The F₃ in patients from the United States, Italy, Germany, and Switzerland were 0.24, 0.20, 0.21, and 0.21, respectively ($\chi^2 = 0.96; P = 0.81$) and those in control subjects were 0.13, 0.19, 0.18, and 0.17, respectively ($\chi^2 = 1.14; P = 0.77$). Moreover, the expected association between APOE genotype and AD was confirmed in the samples from each center (2-sided Fisher exact test, $P ≤ 0.004$; $df = 2$, for each center). Together, these characteristics allowed us to combine the samples and gain sufficient statistical power to test the hypotheses suggested by the results of the initial study. There was a significant association between CST3 B/B and AD ($\chi^2 = 5.37$; 2-sided Fisher exact test, $P = 0.02$). The statistically non-significantly higher F₃ in the patients (21%) than in controls (17%) (Pearson $\chi^2 = 2.61$; $P = 0.11$) was again due to an excess of homozygous carriers of the B allele in the AD group (4.7%) compared with the control subjects (1.3%). The OR for AD in association with B/B in the multicenter sample was 3.87 (95% CI, 1.13-13.21). Regression analysis revealed no significant interaction between APOE and CST3.

**COMBINED SAMPLE**

Analysis of the combined sample of 907 participants confirmed that CST3 was significantly associated with AD, with an OR of 3.8 (95% CI, 1.56-9.25; $P = 0.001$). In the same sample, the ORs for APOE e4 heterozygosity and homozygosity were 3.09 and 6.91, respectively (Table 3). CST3 and APOE independently affected the risk for AD, because the ORs for AD in association with CST3 B/B were similar in APOE e4–negative (3.07; 95% CI, 1.16-8.12; $P = 0.02$) and APOE e4–positive participants. In addition to the independent risks of developing AD, these 2 risk factors combined lowered the age of disease onset. Of all patients with AD, 2.9% carried 2 CST3 B alleles as well as at least 1 APOE e4 allele, compared with none of the 390 control subjects ($\chi^2 = 11.51$; 2-sided Fisher exact test, $P < 0.001$). These patients with AD with both risk factors had a mean±SD age of onset of 69.1±9.5 years compared with 70 years in the overall patient sample.

Results of Kaplan-Meier survival analyses revealed that CST3 B/B reduced mean disease-free survival from 77 years (SE = 0; 95% CI, 76-78 years) in CST3 A/A and A/B patients with AD to 73 years (SE = 2; 95% CI, 70-76 years; log rank $P = 0.05$; $df = 1$). Parallel analyses confirmed the known effects of APOE on mean disease-free survival; in this sample, 80 years in e4–negative (SE = 1; 95% CI, 78-81 years), 73 years in e4–homozygous (SE = 1; 95% CI, 72-75 years), and 70 years in e4–homozygous patients with AD (SE = 1; 95% CI, 68-72 years) (log rank $P < 0.001$; $df = 2$).
The results of this study establish a genetic association between CST3 B/B and AD. There was an excess of the CST3 B/B genotype among patients with AD compared with control subjects in 2 independent study populations. CST3 B/B was present in 4.7% to 9.1% of our patients with AD compared with 10% to 14% APOE ε4/ε4 in this study. In addition, CST3 B/B significantly reduced the average disease-free survival by 4 years. Both effects seemed to be independent of APOE genotype. Together with the absence of CST3 B/B in cognitively normal control subjects older than 74 years, these data indicate that CST3 B/B is a risk factor for late-onset AD. Although statistically significant in this association study, it may be difficult to detect effects of this magnitude with microsatellite markers in whole genome scans of 300 to 600 sib pairs. This difficulty may explain the reported unknown. Cystatin C is initially synthesized with an terminal hydrophobic signal sequence that is removed during synthesis. The Ksp1 polymorphism in CST3 results in an amino acid exchange from alanine to threonine at the –2 position for signal peptidase cleavage (Figure, B). This variation alters the hydrophobicity profile of the signal sequence, and it reduces its ratio of predicted α-helix to β-sheet contents by approximately 42%. This variation could be associated with changes in secretory processing of cystatin C, but our data do not provide information whether such changes are disease-related or the polymorphism tested in this study is in linkage disequilibrium with another disease-related polymorphism upstream or downstream of the analyzed sequence.

Cystatin C is increased in AD brains, along with its high-affinity substrate cathepsin S that is known to cleave APP into β-amyloid peptide (Aβ)–containing derivatives in vitro, and to increase Aβ generation in tissue culture. Cystatin C inhibits cathepsins with a profile that is similar to that of the cysteine protease inhibitor E-64, which is known to differentially affect γ40- and γ42-secretase processing of APP. Further studies are required to test whether cystatin C has similar activities on APP processing, Aβ generation, and amyloid deposition, and whether these differ among the 2 allelic variants.

The Icelandic form of hereditary cerebral hemorrhage with amyloidosis (HCHWA-I) is caused by a leucine-to-glutamine mutation at position 68 in cystatin C. As a result of this mutation, cystatin C amyloid aggregates more readily than the wild-type form. The HCHWA-I is characterized by recurrent strokes and prematurity death before age 40 years and is associated with the deposition in brain blood vessels of cystatin C amyloid. This disease is clearly different from late-onset AD dementia in which there is no point mutation in cystatin C at position 68. Instead, our genotype data provide evidence for a contribution of CST3 B/B as a genetic risk factor to the multifactorial etiology of late-onset AD. Whether CST3 exerts this effect by influencing amyloid deposition, the inflammatory response, or by some other mechanism remains to be determined.

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REFERENCES