Genetic Association of a Cystatin C Gene Polymorphism With Late-Onset Alzheimer Disease

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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 Ksp1 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.

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Alzheimer disease (AD) is the major cause of dementia in the elderly. Familial forms of early-onset AD are caused by heterogeneous mutations in the genes encoding the β-amyloid precursor protein (APP) and the presenilins PS1 and PS2, but the etiology of the much more prevalent forms of late-onset AD is unknown. Several susceptibility genes were reported to influence the risk of developing AD; of these, the effect of the apolipoprotein E gene (APOE) is now uniformly accepted, whereas additional putative genetic risk factors including α2-macroglobulin, interleukin 6, interleukin 1, and cathepsin D are matter of intense debate.

Cystatin C is an endogenous proteinase inhibitor of the cathepsins B, H, L, and S. Cystatin C is a secretory protein, but it also reaches endocytic cellular compartments and inhibits cathepsin activities within the endosomal-lysosomal system.

In the brain, cystatin C is synthesized by neurons, astrocytes, and choroid plexus cells, and its levels increase in response to injury, including ischemia, axotomy, or surgery. Neurochemical concentrations of cystatin C are increased in AD, and cystatin C reportedly colocalizes with β-amyloid in arteriolar walls. The physiological function of cystatin C as a protease inhibitor is regulated either by dimerization or by endoproteolysis mediated by cathepsin D, which is also increased in early endosomes in AD brains.

Cystatin C is also an amyloidogenic protein that accumulates in cortical blood vessels in hereditary cerebral hemorrhage with amyloidosis of both the Dutch and Icelandic types caused by mutations of the APP gene or the cystatin C gene.
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 criteria34; 84% of our patients had Mini–Mental State Examination (MMSE) scores lower than 24, similar to previous studies of AD.35 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 multicentric consortium that included AD research institutions 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.29 The 318–base pair (bp) PCR product generated with primers 024F (TGGGAGGGACGAGGCGTTCC) and 1260R (TCCATGGGCTCTCCACCGAG) was designed to cover all 3 polymorphic KspI sites described above. Digestion with KspI generated 3 fragments of 41, 228, 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.26 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. (CST3), respectively.28 CST3 maps to chromosome 20p11.2; it contains 3 exons, and 2 KspI polymorphisms are known in the 5' untranslated sequence, combined with an additional KspI polymorphism that results in a threonine for alanine substitution at the penultimate position of the signal peptide.29,30 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. 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. The APOE ε4 allele, compared with the control group, was not significantly associated with APOE genotype and AD. Of the patients with AD, 13.6% were homozygous for the ε4 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 ε4 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 ε4 allele (Table 2). Nonconditional logistic regression analyses revealed significant effects on the risk for AD of APOE ε4, 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 ($\chi^2 = 0.96; P = .81$) and those in control subjects were 0.13, 0.19, 0.18, and 0.17, respectively ($\chi^2 = 1.14; P = .77$). Moreover, the expected association between APOE genotype and AD was confirmed in the samples from each center (2-sided Fisher exact test, $P = .004$; $df = 2$, 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, $P = .02$). The statistically nonsignificantly higher F3 in the patients (21%) than in controls (17%) (Pearson $\chi^2 = 2.61; P = .11$) 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; $P = .001$). 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; $P = .02$) 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, $P < .001$). These patients with AD with both risk factors had a mean ± SD age of onset of 69.1 ± 9.5 years compared with 75.0 ± 9.5 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 $P = .05$; $df = 1$). 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–heterozygous (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 $P < .001$; $df = 2$).

### Table 1. CST3 Genotypes in Patients With Alzheimer Disease and Control Subjects in 2 Independent Study Populations*

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Initial AD 110</th>
<th>Control 150</th>
<th>Multicenter AD 407</th>
<th>Control 240</th>
<th>Combined AD 517</th>
<th>Control 390</th>
</tr>
</thead>
<tbody>
<tr>
<td>CST3 A/A</td>
<td>65 (59.1)</td>
<td>99 (66.0)</td>
<td>257 (63.1)</td>
<td>161 (67.1)</td>
<td>322 (62.3)</td>
<td>260 (66.7)</td>
</tr>
<tr>
<td>CST3 A/B</td>
<td>35 (31.8)</td>
<td>48 (32.0)</td>
<td>131 (32.2)</td>
<td>76 (31.7)</td>
<td>166 (32.1)</td>
<td>124 (31.8)</td>
</tr>
<tr>
<td>CST3 B/B</td>
<td>10 (9.1)</td>
<td>3 (2.0)</td>
<td>19 (4.7)</td>
<td>3 (1.3)</td>
<td>29 (5.8)</td>
<td>6 (1.5)</td>
</tr>
</tbody>
</table>

*AD indicates patients with Alzheimer disease. Data are number of participants (percentage of the total number of participants) within the group.

### Table 2. Nonconditional Logistic Regression Analysis Findings for the Effects of APOE e4, CST3 B/B, Sex, and Age on Risk of Alzheimer Disease*

<table>
<thead>
<tr>
<th>Variable</th>
<th>B</th>
<th>SE</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td>CST3 B/B</td>
<td>1.796</td>
<td>0.745</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>APOE e4 positive</td>
<td>1.560</td>
<td>0.302</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Sex</td>
<td>1.027</td>
<td>0.302</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Age</td>
<td>0.056</td>
<td>0.014</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

### Table 3. Odds Ratios for Alzheimer Disease in Association With CST3 or APOE Genotypes Calculated From the Combined Sample*

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Odds Ratio</th>
<th>95% Confidence Interval</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td>CST3 B/B</td>
<td>3.80</td>
<td>1.56-9.25</td>
<td>.001</td>
</tr>
<tr>
<td>APOE e4 positive</td>
<td>3.60</td>
<td>2.71-4.79</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>APOE e4 negative</td>
<td>3.60</td>
<td>2.71-4.79</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Sex</td>
<td>1.027</td>
<td>0.302</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Age</td>
<td>0.056</td>
<td>0.014</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

*The combined sample includes 517 patients and 390 controls.
†Fisher exact test.
‡APOE e4 homozygotes excluded.
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 e4/e4 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 absence of positive LOD scores at chromosome 20p11.2, the genomic region of CST3.

Of the susceptibility genes reported to influence the risk of developing AD, the effects of the APOE e4 allele are best known and accepted. Carrying the e4 allele increases the risk of developing AD and lowers the age of dementia onset in a dose-dependent fashion, whereas the e2 allele may protect against AD, or at least delay its onset. The mechanisms by which APOE affects AD are unknown, although there is an e4 dose-dependent increase in brain amyloid deposits, and amyloid plaques are reduced in transgenic mice lacking APOE. APOE genotypes do not influence the rate of cognitive decline once dementia sets in. Because the influence of APOE is so pervasive in AD, we determined the APOE genotypes of our patients and control subjects to distinguish APOE from CST3 effects. In doing so, we confirmed in our populations’ prior reports that the APOE e4 allele was associated with sporadic late-onset AD, and that APOE e4 shortened the mean dementia-free survival time. We also documented that the association of the CST3 B/B genotype with sporadic late-onset AD was independent of APOE, as indicated by a similar OR of CST3 B/B for AD in APOE e4–negative subjects compared with APOE e4–positive participants. Because 2.9% of our patients with AD but none of the control subjects were APOE e4–positive CST3 B/B carriers, additive effects of APOE and CST3 cannot be excluded in this group of patients.

In contrast to APOE e4, the heterozygous B allele did not reduce mean age of onset or survival. It is therefore possible that the effects of the CST3 B allele on the risk of AD followed a recessive pattern, suggesting that CST3 B may represent a recessive risk allele for late-onset AD. In the participants of this study, the OR of CST3 B/B for AD increased with advancing age from 2.59 at ages younger than 75 years to 8.78 in the age group 75 years and older, suggesting that the risk of AD attributable to CST3 B/B reaches its maximum at an older age than the risk attributable to APOE e4, and that CST3 B/B is a risk factor particularly for late-onset AD. This pattern differs from the effects of APOE, which peak in the seventh and eighth decades of life and then decline thereafter.

The role of cystatin C in the pathogenesis of AD is unknown. Cystatin C is initially synthesized with a terminal hydrophobic signal sequence that is removed during synthesis. The KspI 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 premature 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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