The Cathepsin D Gene Exon 2 (C224T) Polymorphism and Sporadic Alzheimer’s Disease in European Populations

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The cathepsin D gene (CTSD) exon 2 (C224T) polymorphism has been associated with an increased risk for sporadic Alzheimer’s disease (AD), but with controversial findings. We studied CTSD exon 2 (C224T) and apolipoprotein E (APOE) genotype frequencies in 168 AD patients and 218 age-matched healthy controls from Southern Italy. No statistically significant differences were found in CTSD allele or genotype frequencies between AD patients and controls, and there were no interactions with sex or APOE genotype. Furthermore, comparing our results with the findings from other European populations, the CTSD*T allele frequency showed a statistically significant increasing trend from Northern to Southern regions of Europe in AD patients and controls (z = 2.51, p < .01; z = 4.02, p < .001, respectively), with a concomitant inverse trend for CTSD*C allele frequency. The regional differences in CTSD allele frequencies could be related to the different patterns of association between this polymorphism and AD in various European studies.

CATHEPSIN D (catD) is an intracellular aspartyl protease that exhibits beta and gamma secretase-like activity in vitro by cleaving amyloid precursor protein into beta-amyloid (Aβ) peptide (1–4). CatD was also shown to degrade tau protein into fragments corresponding in mass to those found in neurofibrillary tangles (5–7). The gene encoding catD in humans (CTSD) is located in the short arm of chromosome 11 (11p15.5). The CTSD gene has a T/C polymorphism located at position 224 in exon 2, named also as SNP (rs17571) that results in an aminoacid change (Ala58Val).

Initially, Papassotiropoulos and colleagues (8,9) reported the results of two independent case-control studies analyzing this polymorphism both of which found a highly significant overrepresentation of the CTSD*T allele in Alzheimer’s disease (AD) patients versus controls. In addition, carriers of both the CTSD*T allele for CTSD and at least one ε4 allele for APOE were reported to be almost 20 times more likely to have AD than were noncarriers of these alleles (9). However, several other studies (10–22) attempted to confirm the association between the CTSD exon 2 (C224T) polymorphism and AD yielding contrasting findings. Furthermore, Papassotiropoulos and colleagues (23) recently provided evidence that CTSD*T allele is related to low cerebrospinal fluid levels of Aβ peptide 1-42 Aβ(42) in AD patients, possibly indicating increased deposition of Aβ in the brain or reduced Aβ production as a result of decreasing numbers of surviving neurons. Finally, the CTSD exon 2 (C224T) polymorphism was recently reported to be significantly associated with general intelligence in healthy elderly people (24).

The first aim of the present study was to investigate whether there was evidence in Southern Italy to support previous findings (8,9) of an association between the CTSD exon 2 (C224T) polymorphism and increased risk of AD in a sample of sporadic, early- and late-onset AD patients and age- and sex-matched controls. Secondly, we explored a possible effect of geographic genetic variations on existing reported associations comparing our results with the findings from published studies on other European populations. Finally, we examined the putative interaction between the CTSD exon 2 (C224T) polymorphism and apolipoprotein E (APOE) allele and sex strata, given the recent findings of a higher prevalence of the APOEε4 allele and a lower prevalence of the APOEε2 allele in Italian AD patients than in nondemented controls, suggesting a role of this polymorphism in AD also in Italy (25).

METHODS

Patients

We studied a total sample of 386 participants from Apulia (Southern Italy). The AD group consisted of 168 patients (109 women and 59 men, mean age at onset: 70.1 ± 10.4 standard deviation [SD] years) including 100 patients with sporadic late-onset AD [age at onset ≥ 70 years; mean actual age 77.1 ± 5.2 SD years; 61 women and 39 men],
and 68 patients with sporadic early-onset AD (age at onset < 70 years; mean actual age 59.8 ± 6.8 SD years; 48 women and 20 men), consecutively examined in the Centre for Aging Brain, Memory Unit, Department of Geriatrics, Bari University Hospital, Italy, between June 1998 and April 2004. We used 70 years as the age cutoff to ensure homogenous age groups, and we adopted this convention also in other recent studies (26–28). The age at onset of AD symptoms was estimated by semistructured interviews with the patients’ caregivers (29). Clinical diagnosis of probable AD was made according to the National Institute for Neurological and Communicative Disorders and Stroke/Alzheimer’s Disease and Related Disorders Association criteria (30). To be eligible for inclusion in this study, patients were required to have a clinical dementia rating (CDR) scale (range, 0–5) score of 0.5 or higher (31), a modified Hachinski ischemic score (range, 0–12) less than 3 (32), and a Hamilton depression scale (range, 0–67) score less than 17 (33).

The nondemented age-, sex-, and ethnicity-matched control group included 218 unrelated caregivers (147 women and 71 men, mean age 75.5 ± 8.5 SD years) spouses, friends, neighbors, or volunteers, consecutively examined between June 1998 and April 2003 in our Centre. This group included 183 participants with a mean actual age of 77.9 ± 5.3 SD years (124 women and 59 men), and 35 participants with a mean actual age of 62.9 ± 10.7 SD years (23 women and 12 men), with no known diagnosis of dementia or other chronic neurological diseases or psychiatric syndromes with cognitive impairment, cerebrovascular disease, nephropathy, or end-stage renal disease, and no severe functional limitations. The ascertainment, diagnosis, and collection of cases and controls have been described in detail elsewhere (29). After complete description of the study, written informed consent was obtained from all participants and/or their relatives. The study protocol has received the approval of the Ethical Committee of the University of Bari.

CTSD Exon 2 (C224T) and APOE Polymorphism Genotyping

Genomic DNA was extracted from peripheral blood samples by the GFXa Genomic Blood DNA Purification Kit (Amersham Biosciences, Piscataway, NJ). The CTSD exon 2 (C224T) polymorphism genotyping was performed on a LightCycler system (Roche, Mannheim, Germany) by melting curve analysis using a fluorescein isothiocyanate (FITC)-labeled sensor (5’-ACGCGCTGGCACCACCTGGX 3’) and LightCycler Red 640 anchor (5’-AGTACTTGA GACGGGCGCCTTGCCAP-3’; TibMol-Biol (Genova, Italy) probes. The primer used in the amplification were forward primer, 5’-TCCGCTGCAACAGTTCAGCTC-3’ and reverse primer, 5’-CGTTCGACGAGGCCTAA GACC-3’. The following 20 µl reaction mix was used for polymerase chain reaction amplification: 100 ng of genomic DNA, 50 pmol of each primer, 3 pmol of each probe, 3.5 mM MgCl2, 1 × DNA Master Hybridization Probes (Roche Diagnostics, Mannheim, Germany). The polymerase chain reaction “touchdown” conditions, to reduce mispriming and to increase efficiency, included an initial denaturation of 2 minutes at 95°C, then 45 cycles of 10 seconds at 94°C, five cycles of 15 seconds at 62°C, followed by a 0.4°C decrease of the annealing temperature every second cycle up to 55°C, 20 seconds at 72°C, followed by melting curve analysis program with denaturation of 30 seconds at 94°C, probes annealing for 60 seconds at 40°C, slow raising temperature up to 90°C to permit melting of the detection probes, and final cooling at 40°C. The melting temperatures were 62°C for allele C and 68°C for allele T. APOE genotypes were determined as described elsewhere (25).

Statistical Analysis

The statistical analysis was performed with the Pearson chi-square test to make genotype and allele comparisons as well as to test for agreement of data with Hardy–Weinberg principles. Allele frequencies were determined by allele counting. To express variances of the allele frequencies, 95% confidence intervals were used, the upper and lower values of which were calculated according to the Wilson formulas. The results of statistical inference were adjusted according to Bonferroni inequality. In this analysis we used the chi-square value corresponding to 0.05/3 = 1.7 percent for each of the individual comparisons. To evaluate whether the association between CTSD alleles and AD were homogeneous in all APOE alleles and sex strata we used a logistic model. Difference in mean age at onset between AD patients who inherited at least one T allele and T-noncarriers was evaluated by Student t test for unpaired data. The Cochran–Armitage trend test was carried out to evaluate the possible geographic trend among the CTSD allele frequencies in AD patients and controls of the present study and in eight other European populations from Sweden, Northern Ireland, Germany (two populations), Poland, Northern Spain, and Central Italy (two populations) from published studies (8–10,14,16–19). We excluded the genotypes from the calculation of geographical trend for the lack in almost all the studies of persons bearing the CTSD*T/T* genotype. Criteria for selection of published studies on the association of the CTSD exon 2 (C224T) polymorphism and AD were a sample amplitude > 100, the report of allele or genotype frequencies, a group of controls age- and sex-matched with AD cases, and the diagnosis of AD made according to the same clinical criteria (30).

Statistical analyses were conducted with Statistical Analysis System software (version 8.2; SAS Institute, Cary, NC).

RESULTS

Table 1 shows CTSD allele and genotype frequencies in AD patients and controls in Southern Italy. The distribution of CTSD exon 2 (C224T) and APOE genotypes and alleles in AD cases and controls followed Hardy–Weinberg equilibrium (CTSD AD patients: Pearson X² = 0.157, p = .692; CTSD controls: Pearson X² = 2.051, p = .152; APOE AD patients: Pearson X² = 1.622, p = .654; APOE controls: Pearson X² = 4.211, p = .240). No statistically significant differences were found in CTSD genotype and allele frequencies between AD patients and controls in this Southern Italian population, when we considered population on the whole (CTSD*C/C vs CTSD*C/ *T and CTSD*T/T: Pearson X² = 0.000, df = 1, Bonferroni adjusted p = 1.000).
ferroni-\( p = 1 \); CTSD*C/T vs CTSD*C/C and CTSD*T/T; Pearson X^2 = 0.100, df = 1, Bonferroni-\( p = 1 \); CTSD*T/T vs CTSD*C/T and CTSD*C/C; Pearson X^2 = 1.140, df = 1, Bonferroni-\( p = 0.870 \); CTSD*T vs CTSD*C; Pearson X^2 = 0.090, df = 1, Bonferroni-\( p = 1 \).

No statistically significant differences in genotype and allele distribution between AD cases and healthy controls were observed when the AD group was categorized by age at onset into early- and late-onset AD subsets. Furthermore, we did not find any statistically significant differences in rates between CTSD alleles and AD controlling for APOE alleles and sex strata (Table 2). Participants who had inherited at least one T allele had a mean age at onset that was about 2.0 years higher than did participants who were noncarriers of the T allele, although this was not significant (Student \( t \) test for unpaired data: 1.07, \( p = 0.310 \)).

It is interesting that, in the present study, the CTSD*T allele frequency showed a statistically significant increasing trend from Northern to Southern regions of Europe in AD patients and controls (\( z = 2.52, p < 0.01 \); \( z = 4.04, p < 0.001 \), respectively), with a concomitant inverse trend for CTSD*C allele frequency (Table 3).

**DISCUSSION**

In the present study, we did not find in Southern Italy any evidence of association of the CTSD exon 2 (C224T) polymorphism with AD, nor in early- and late-onset subsets of AD patients, in contrast to the association between this polymorphism and AD risk in APOEe4 carriers initially reported by Papassotriopoulos and colleagues (8,9), and subsequently replicated by other authors with controversial findings (10–22). However, comparing our results with the findings of several other European studies from Northern Spain (14), Poland (16), Sweden (17), Central Italy (18,19), and Scotland (20), and with the studies on Japanese (15), and Han Chinese (21) populations in which no increased AD risk was found to be associated with CTSD exon 2 (C224T) polymorphism. Recently, Crawford and colleagues (22) examined their Caucasian/Hispanic populations and populations from previous studies (8–10). They found evidence that CTSD gene does contribute risk for AD, but this contribute is small (odds ratio: 1.92), and their reanalysis of the results of Mclllroy and colleagues (10) revealed a significant association between the CTSD*T allele and AD (\( p = 0.04 \)) in this Northern Ireland population.

### Table 1. Cathepsin D Gene (CTSD) Exon 2 (C224T) Genotype and Allele Distributions in Alzheimer’s Disease (AD) Patients and Nondemented Age- and Sex-Matched Controls in a Southern Italian Population

<table>
<thead>
<tr>
<th>Participants</th>
<th>Genotypes, N Frequency (95% CI)</th>
<th>Alleles, N Frequency (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Age at Onset and at Collection (SD)</td>
<td>CTSD*C/C</td>
</tr>
<tr>
<td>AD patients (( n = 168 ))</td>
<td>70.1 (10.4)</td>
<td>0.138</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.82 (0.87–0.76)</td>
</tr>
<tr>
<td>Controls (( n = 218 ))</td>
<td>75.5 (8.5)</td>
<td>0.179</td>
</tr>
<tr>
<td>AD patients ( \geq 70 ) y (( n = 100 ))</td>
<td>77.1 (5.2)</td>
<td>0.80 (0.87–0.71)</td>
</tr>
<tr>
<td>Italian controls ( \geq 70 ) y (( n = 183 ))</td>
<td>77.9 (5.3)</td>
<td>0.80 (0.87–0.76)</td>
</tr>
<tr>
<td>AD patients &lt; 70 y (( n = 68 ))</td>
<td>59.8 (6.8)</td>
<td>0.58</td>
</tr>
<tr>
<td>Italian controls &lt; 70 y (( n = 35 ))</td>
<td>62.9 (10.7)</td>
<td>0.29</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genotypes, N Frequency (95% CI)</th>
<th>Alleles, N Frequency (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTSD*C/C</td>
<td>CTSD*C/T</td>
</tr>
<tr>
<td>AD patients (( n = 168 ))</td>
<td>70.1 (10.4)</td>
</tr>
<tr>
<td>Controls (( n = 218 ))</td>
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<tr>
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<td>62.9 (10.7)</td>
</tr>
</tbody>
</table>

**Note:** \( n \) = Number of individuals genotyped; SD = standard deviation; CI = confidence interval.

### Table 2. Cathepsin D Gene (CTSD) Exon 2 (C224T) Allele Distributions According to Apolipoprotein E (APOE) Allele Presence and Sex in Alzheimer’s Disease (AD) Patients and Nondemented Age- and Sex-Matched Controls in a Southern Italian Population

<table>
<thead>
<tr>
<th>Participants</th>
<th>Sex</th>
<th>APOE Alleles</th>
<th>CTSD*C</th>
<th>CTSD*T</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD patients (( n = 168 ))</td>
<td>Men</td>
<td>( e2 )</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>( e3 )</td>
<td>173</td>
<td>19</td>
<td>0.90 (0.95–0.85)</td>
</tr>
<tr>
<td></td>
<td>( e4 )</td>
<td>32</td>
<td>4</td>
<td>0.89 (0.96–0.75)</td>
</tr>
<tr>
<td></td>
<td>Women</td>
<td>( e2 )</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>( e3 )</td>
<td>309</td>
<td>29</td>
<td>0.91 (0.94–0.88)</td>
</tr>
<tr>
<td></td>
<td>( e4 )</td>
<td>75</td>
<td>9</td>
<td>0.89 (0.94–0.81)</td>
</tr>
<tr>
<td>Controls (( n = 218 ))</td>
<td>Men</td>
<td>( e2 )</td>
<td>28</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>( e3 )</td>
<td>218</td>
<td>22</td>
<td>0.91 (0.94–0.87)</td>
</tr>
<tr>
<td></td>
<td>( e4 )</td>
<td>18</td>
<td>2</td>
<td>0.90 (0.97–0.70)</td>
</tr>
<tr>
<td></td>
<td>Women</td>
<td>( e2 )</td>
<td>43</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>( e3 )</td>
<td>455</td>
<td>51</td>
<td>0.90 (0.92–0.87)</td>
</tr>
<tr>
<td></td>
<td>( e4 )</td>
<td>24</td>
<td>2</td>
<td>0.92 (0.98–0.76)</td>
</tr>
</tbody>
</table>

**Note:** \( n \) = Number of individuals genotyped; CI = confidence interval.
Our analysis on the possible influence of CTSD genotype on the age of onset of AD showed that patients who had inherited at least one T allele had a mean age at onset that was about 2.0 years higher than did participants who were noncarriers of the T allele, although this was not significant. The present findings confirm those on Northern Spanish (14) and Han Chinese (21) populations, in which no significant variation existed in the age of onset for developing AD between CT and TT and CC genotypes.

At present, 10 studies (8,9,11,13,14,17–21) obtained data on both CTSD and APOE genotypes, but only four reports found some evidence of an interaction (8,9,11,21). Menzer and colleagues (11) suggested that there might be a stronger effect of the T allele of CTSD on AD risk in males, and an interaction between the T allele of CTSD and the e4 allele of APOE in males but not in females, in their sample of patients from Germany, Switzerland, and Northern Italy. However, we did not find any statistically significant differences in frequencies between CTSD alleles and AD among APOE and sex strata.

Very recently, in a large meta-analysis, 14 studies probing the relation between the CTSD exon 2 (C224T) polymorphism and AD susceptibility were identified (8–20,22), with a total of 3175 AD cases and 3334 controls. There was a trend suggesting that the CTSD*T allele may confer increased susceptibility to AD. The summary odds ratio was 1.17 by random effects (p = .14), and no evidence of an association of the CTSD*TT genotype with the risk of AD relative to the CTSD*CC genotype was found (35). Furthermore, among carriers of the T allele, the presence of APOEe4 increased the risk of AD 6.07-fold, and among persons without the T allele, the presence of APOEe4 increased the risk of AD 4.09-fold. Therefore, the meta-analysis of Ntais and colleagues (35) suggested that the CTSD exon 2 (C224T) polymorphism is not a major risk factor for AD, although a small effect or an enhancement of the APOEe4 effect cannot be excluded.

The possible explanations for these conflicting results are, at present, unclear. Discrepancies between genetic association studies are common and may be ascribed to different causes: the initial finding may be false-positive due to multiple testing or population heterogeneity, negative studies may result from low statistical power or population mixture, or the initial finding may hold true only in certain...
populations. The discrepant findings on the possible association of CTSD exon 2 (C224T) polymorphism with AD could reflect true genetic differences, as the study populations were drawn from different countries (Sweden, Germany, Northern Ireland, Poland, Northern Spain, Central Italy, United States, Japan, and China) (8–22). Moreover, the very small number of CTSD*TT carriers in almost all the populations studied suggests a potential statistical bias that can occur by chance. Ethnic differences have also previously been documented in a study on American Caucasians and Hispanics, with a tendency towards an increase in T-carrying genotypes in cases versus controls in the Caucasian sample, and the opposite trend in Hispanics (22). In contrast, Matsu and colleagues (15) found no significant association between the CTSD exon 2 (C224T) polymorphism and AD in the different ethnic groups examined (Japanese and American Caucasians).

It is also possible that there is another nearby locus associated with increased risk of AD that is in linkage disequilibrium with the T allele of the CTSD gene in German populations (8,9), but not in other ethnic groups such as Italians or Spaniards (14,18,19). It is noteworthy that a candidate gene to be linked to the CTSD locus is the gene for FE65 (36). Both FE65 and CTSD are located on the short arm of chromosome 11 (11p15), and both proteins are involved in amyloid precursor protein metabolism. However, no risk-modifying interaction and no linkage disequilibrium between these genes have been recently observed in a German population (37).

Finally, comparing our results with the findings from other published European populations, the CTSD*T allele frequency in AD patients and controls showed a statistically significant increasing trend from Northern to Southern regions of Europe, whereas there was a concomitant decrease in CTSD*C allele frequency. A possible explanation for the conflicting findings that have been reported (8–22) could be linked to the variability in the strength of association that may exist between the CTSD exon 2 (C224T) polymorphism and AD which could be modified by European regional differences in allele frequencies, as we have identified in AD patients for the APOE (34), the less density lipoprotein receptor-related protein 1 (26), and the interleukin-6–174 G/C promoter polymorphism genes (39). Thus, it appears likely that CTSD exon 2 (C224T) gene polymorphism may be a susceptibility factor for AD, though it exerts only small effects in the general population which are detectable in some study samples (8–10) but not in others (11–22), requiring in some geographical regions larger sample sizes to detect the association with the disease.

Conclusion

In Southern Italy no significant differences were found in CTSD exon 2 (C224T) allele or genotype frequencies between AD patients and controls, or in early- and late-onset subsets of AD patients, and without interactions with sex or APOE genotype. Given the controversial association of the CTSD exon 2 (C224T) polymorphism with AD, we suggest that the regional variations in allele frequencies shown in AD and controls in the present study could be related to the different patterns of association between this polymorphism and the disease in various European populations, suggesting the need for further studies on larger samples, controlling for ethnic and geographic variability.

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