SHORT REPORT

Genetic associations between cathepsin D exon 2 C→T polymorphism and Alzheimer's disease, and pathological correlations with genotype

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Genetic variations represent major risk factors for Alzheimer's disease (AD). While familial early onset AD is associated with mutations in the amyloid precursor protein and presenilin genes, only the e4 allele of the apolipoprotein E (*APOE*) gene has so far been established as a genetic risk factor for late onset familial and sporadic AD. It has been suggested that the C→T (224Ala→Val) transition within exon 2 of the cathepsin D gene (*CTSD*) might represent a risk factor for late onset AD. The objective of this study was to investigate whether possession of the *CTSD* exon 2 T allele increases the risk of developing AD, and to determine whether this modulates the amyloid pathology of the disease in conjunction with, or independent of, the *APOE* e4 allele. Blood samples were obtained from 412 patients with possible or probable AD and brain tissues from a further 148 patients with AD confirmed by postmortem examination. *CTSD* and *APOE* genotyping were performed by PCR on DNA extracted from blood, or from frontal cortex or cerebellum in the postmortem cases. Pathological measures of amyloid β protein (Aβ), as plaque $A\beta_{40}$ and $A\beta_{42(3)}$ load and degree of cerebral amyloid angiopathy were made by image analysis or semiquantitative rating, respectively. *CTSD* genotype frequencies in AD were not significantly different from those in control subjects, nor did these differ between cases of early or late onset AD or between younger and older controls. There was no gene interaction between the *CTSD* T and *APOE* e4 alleles. The amount of plaque $A\beta_{40}$ was greater in patients carrying the *CTSD* T allele than in non-carriers, and in patients bearing *APOE* e4 allele compared with non-carriers. Possession of both these alleles acted synergistically to increase levels of plaque $A\beta_{40}$, especially in those individuals who were homozygous for the *APOE* e4 allele. Possession of the *CTSD* T allele had no effect on plaque $A\beta_{42(3)}$ load or degree of CAA. Possession of the *CTSD* T allele does not increase the risk of developing AD per se, but has a modulating effect on the pathogenesis of the disorder by increasing, in concert with the *APOE* e4 allele, the amount of Aβ deposited as senile plaques in the brain in the form of $A\beta_{40}$.

Although initial reports[1 2] described a strong association between the C→T (224Ala→Val) transition in exon 2 of the cathepsin D gene (*CTSD*) and Alzheimer's disease (AD), this finding remains to be replicated.[3–6] Indeed, a recent meta-analysis[7] concluded that the exon 2 *CTSD* polymorphism is not a major risk factor for AD, although it might, as a disease modifying factor, enhance the effects of the apolipoprotein E (*APOE*) e4 allele.

Cathepsin D is an aspartyl protease that, in vitro, can cleave amyloid precursor protein into amyloid β protein (Aβ) at both the β-secretase[8 9] and γ-secretase[10] sites. Being functional,[11] the exon 2 *CTSD* C→T polymorphism might modulate the course of AD by increasing Aβ precursor protein (APP) breakdown, thereby promoting Aβ pathology. In this study, we investigated whether the exon 2 *CTSD* T allele influences the pathological phenotype of AD by modulating brain amyloid plaque load or extent of cerebral amyloid angiopathy (CAA).

MATERIALS AND METHODS

DNA was extracted by routine methods from 2 ml EDTA blood samples taken from 414 patients with AD. These patients were ascertained either through clinical old age psychiatry (OAP) services in Manchester (100 individuals) with diagnosis of AD being made according to DSM III-R Criteria,[13] or through outpatient clinics at the cerebral function unit (CFU) of Greater Manchester Neurosciences Centre, Hope Hospital (314 individuals) in whom diagnosis of AD was consistent with NINCDS-ADRDA criteria.[14] DNA was also extracted from frozen cerebral cortex or cerebellum from a further 146 individuals who had died from AD, verified by postmortem examination according to CERAD Criteria.[15] Of these patients, 92 had been identified through the CFU, 45 through OAP services, and 9 through tissue donations under the auspices of the Alzheimer's Society (UK). Twelve of the deceased patients had been investigated in the CFU during their lifetime but were not doubly represented within the postmortem examination group. AD cases were stratified into early onset AD (EOAD; 317 patients, mean (SD) age at onset 56.5 (5.6) years) and late onset AD (LOAD; 243 patients, mean age at onset 72.8 (5.9) years) groups. Control data were derived from blood samples taken from a cohort of 767 mentally normal people aged >50 years resident within the same Greater Manchester region from which the patients with AD were drawn.[16] All subjects were white. All blood and brain samples were collected with the approval of the local ethics research committee.

The exon 2 *CTSD* C→T polymorphism and *APOE* genotype were determined by PCR as described previously.[16 17] Given a *CTSD* T allele frequency of 0.1, there was at least 80% power to detect an effect size of 1.6 or greater for AD and control

Abbreviations: Aβ, amyloid beta; AD, Alzheimer's disease; APP, amyloid β precursor protein; CAA, cerebral amyloid angiopathy; CFU, cerebral function unit; CTSD, cathepsin D; EOAD, early onset AD; LOAD, late onset AD; OAP, old age psychiatry; SNP, single nucleotide polymorphism

cohorts, an effect size of 1.8 or greater for the EOAD cohort and younger controls, and an effect size of 2.1 or greater for the LOAD cohort and the older controls.

Brain levels of $A\beta_{40}$ or $A\beta_{42(3)}$ were measured by image analysis following immunohistochemical staining of paraffin wax embedded sections of frontal cortex (thickness 6 μm) using BA27 and BC05 antibodies for $A\beta_{40}$ or $A\beta_{42(3)}$, respectively, and the extent of CAA within the brain was assessed semiquantitatively in Aβ immunostained sections using 4G8 antibody (Signet Laboratories, Waltham MA), as previously described.[18 19]

Statistical analysis

Logistic regression analysis was performed using Stata (2001) software to determine any synergistic interaction between *APOE* alleles and the *CTSD* C→T polymorphism.

RESULTS

CTSD genotype and allele frequencies for AD and control subjects are given in table 1. The frequencies of the *CTSD* CC, CT, and TT genotypes did not differ significantly between the AD and control groups ($\chi^2 = 3.8$; $p = 0.148$), nor did the frequency of T allele carriers (that is, the total number of individuals with CT or TT genotype) ($\chi^2 = 0.03$; $p = 0.867$). However, there was a trend for the TT genotype to be more common in AD than controls (Fisher's exact test, $p = 0.077$). There were no significant differences between *CTSD* genotype frequencies when patients with AD were stratified into EOAD and LOAD groups ($\chi^2 = 0.1$; $p = 0.743$), when patients with EOAD were compared with younger control subjects ($\chi^2 = 2.3$; $p = 0.321$), or when patients with LOAD were compared with older control subjects ($\chi^2 = 1.5$; $p = 0.463$). As expected, the proportion of people bearing at least one *APOE* e4 allele was significantly higher ($\chi^2 = 34.8$; $p<0.001$) in the AD group (63%) than in the control group (27%). There was no synergistic interaction between the *CTSD* C→T polymorphism and the *APOE* e4 allele. *CTSD* genotype frequencies were not significantly different between *APOE* e4 allele carriers and non-carriers in the whole AD cohort, or when stratified into EOAD and LOAD groups (data not shown).

In the deceased AD patients, there were no significant differences in the amount of plaque $A\beta_{42(3)}$ or extent of CAA within the brains of *CTSD* T allele carriers compared with C allele carriers, or according to *APOE* genotype, or between carriers and non-carriers of *APOE* e4 allele (table 2). However, the amount of plaque $A\beta_{40}$ differed significantly between *APOE* genotype groups ($F_{5,127} = 11.2$; $p<0.001$), being significantly greater ($p<0.001$) in *APOE* e4 allele carriers than in non-carriers (table 2). Furthermore, plaque $A\beta_{40}$ was also significantly greater ($p = 0.006$) in *CTSD* T allele carriers than in non-carriers (table 2). Most importantly, levels of plaque $A\beta_{40}$ levels differed significantly across the 4 *APOE* e4 allele/*CTSD* T allele combination groups ($F_{3,127} = 7.6$; $p<0.001$); possession of *CTSD* T allele significantly augmented the level of $A\beta_{40}$ in both carriers and non-carriers of *APOE* e4 allele (table 2). Indeed, when individuals homozygous for *APOE* e4 allele were investigated separately, not only was plaque $A\beta_{40}$ higher in *CTSD* T allele carriers (10.5 (5.1)) than in non-carriers (6.8 (4.5)), but those individuals had the highest $A\beta_{40}$ levels of any of the *APOE* e4 allele/*CTSD* T allele combination groups. Because of the relatively small number of patients involved, it was not possible to confirm this finding statistically.

Table 2 Pathological measures stratified according to *APOE* and *CTSD* genotypes and alleles, separately and in combination

	$A\beta_{40}$	$A\beta_{42}$	CAA
APOE genotype			
e2/e2 (1)	1.2	15.2	9.0
e2/e3 (8)	2.2 (1.7)	10.2 (5.4)	6.3 (1.5)
e2/e4 (2)	5.4 (2.4)	11.2 (0.7)	6.5 (3.5)
e3/e3 (48)	2.2 (2.2)	9.6 (4.9)	7.3 (2.5)
e3/e4 (60)	3.2 (2.7)	9.8 (5.3)	7.1 (3.1)
e4/e4 (27)	7.8 (5.0)	9.8 (3.1)	8.7 (2.8)
e4− (57)	2.1 (2.1)	9.8 (5.0)	7.3 (2.4)
e4+ (89)	4.5 (4.0)	9.8 (4.7)	7.5 (3.1)
CTSD genotype			
CC (127)	3.3 (3.2)	9.7 (4.8)	7.5 (2.9)
CT (19)	5.9 (5.2)	10.5 (4.1)	7.1 (2.4)
APOE/CTSD combination			
e4− T− (77)	2.1 (2.0)	9.4 (4.8)	7.4 (2.4)
e4− T+ (6)	3.8 (2.5)	12.0 (5.5)	6.5 (3.1)
e4+ T− (50)	4.1 (3.5)	9.8 (4.9)	7.6 (3.2)
e4+ T+ (13)	7.0 (5.9)	9.7 (3.3)	7.3 (2.2)

Data are means (SD).

DISCUSSION

In this study, *CTSD* genotype and allele frequencies in AD did not differ from controls in either EOAD or LOAD (see also Ntais *et al*[7]). Although it has been suggested[1 2 6 7] that possession of *CTSD* T allele might confer a slightly increased risk of AD through enhancement of *APOE* e4 allelic effects, no (genetic) interaction between *CTSD* T allele and *APOE* e4 allele either overall, or in EOAD and LOAD was found (see also Bhoja *et al*, Crawford *et al*, Ingegni *et al*[3–5]).

Consistent with previous reports,[20 21] plaque $A\beta_{40}$ was increased in *APOE* e4 allele carriers. Importantly, plaque $A\beta_{40}$ was also increased in *CTSD* T allele carriers, and there was a synergistic effect between *APOE* e4 allele and *CTSD* T allele; carriers of both had a greater deposition of $A\beta_{40}$ than carriers of either allele alone, or non-carriers of both. Consequently, the highest levels of $A\beta_{40}$ were present in patients carrying the *CTSD* T allele who were also homozygous for the *APOE* e4 allele. *CTSD* T allele did not influence plaque $A\beta_{42(3)}$ load or extent of CAA (see above) or phosphorylated tau protein, degree of astrocytosis and microgliosis, or vessel arteriosclerosis (unpublished data).

The mechanism underlying this biological synergy is unclear. The APOE E4 isoform may reduce the threshold

Table 1 *CTSD* genotype and allele frequencies in 560 AD and 767 control subjects

		CTSD genotypes, n (%)			Alleles	
Cohort	n	CC	CT	TT	C	T
All AD	560	475 (84.9)	79 (14.1)	6 (1.1)	1029 (91.9)	91 (8.1)
EOAD	317	270 (85.1)	44 (13.6)	3 (1.0)	574 (92.2)	50 (7.8)
LOAD	243	205 (84.3)	35 (14.4)	3 (1.3)	445 (91.6)	41 (8.4)
All controls	767	648 (84.5)	117 (15.3)	2 (0.3)	1413 (92.0)	121 (8.0)
Younger controls	496	422 (85.1)	73 (14.7)	1 (0.2)	917 (92.4)	75 (7.6)
Older controls	270	225 (83.3)	44 (16.3)	1 (0.4)	494 (91.5)	46 (8.5)

The AD group was stratified into EOAD and LOAD cohorts and the control group into younger and older control groups.

for fibrillisation of Aβ, permitting a greater seeding of $A\beta_{40}$ upon pre-existing deposits of the more highly fibrillogenic $A\beta_{42}$. Another possibility, as suggested by studies in transgenic mice,[22] is that *APOE* e4 allele carriers, especially homozygous individuals, have a reduced ability to clear Aβ from the brain compared with homozygous and heterozygous carriers of *APOE* e3 allele. The T allelic variant of *CTSD* is functionally more active than the C allelic form,[11] and the protein isoform transcribed by T allele may catabolise APP more readily at the β-secretase and γ-secretase sites to yield greater amounts of both $A\beta_{40}$ and $A\beta_{42}$.[8–10] This might lead to an increased availability of $A\beta_{40}$ within the extracellular space, which could enable a greater degree of seeding of this particular isoform of Aβ upon a nidus of pre-existing $A\beta_{42}$ than is possible when the APOE E4 isoform alone is present.

One limitation of the present study is that only a single single nucleotide polymorphism (SNP) within *CTSD* was investigated. There are at least 18 other SNPs in *CTSD* (see Majores *et al*[12] and www.ncbi.nlm.nih.gov). Two silent mutations occur in exons 3 and 4, and there are two polymorphisms in introns 5 and 8.[12] Other SNPs occur in the untranslated reading frame. However, we focused upon the exon 2 SNP for two reasons. Firstly, because this polymorphism is known to be functional in a manner biologically relevant to AD.[11] Secondly, the exon 2 coding polymorphism is in linkage disequilibrium with both SNPs in exons 3 and 4, and with the SNP in intron 8, at least, generating two haplotypes.[12] Although the SNP in intron 5 generates a further haplotype, this SNP is not considered to be functional, at least in terms of influencing RNA splicing.[12] We believe our conclusion relating to the biological effect of *CTSD* exon 2 polymorphism on amyloid pathology is valid, although we have not examined potential effects of other variations in *CTSD*.

Future work should assess the effects of *CTSD* haplotype on amyloid pathology in order to elucidate the genetic elements responsible for exacerbating this facet of AD pathology.

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