The Association of a Cystatin C Gene Polymorphism with Late-Onset Alzheimer's Disease and Vascular Dementia

C. Lin[1], S-T. Wang[2], C-W. Wu[3], L-J. Chuo[4], and Y-M. Kuo[3,*]

[1]*Department of Physiology*
China Medical College
Taichung, Taiwan
[2]*Departments of Public Health*
[3]*Departments of Cell Biology and Anatomy, College of Medicine*
National Cheng Kung University
Tainan, Taiwan
[4]*Department Psychiatry*
Taichung Veterans General Hospital
Taichung, Taiwan

Abstract

A polymorphism in the cystatin C (*CST3*) gene was suggested to associate with Alzheimer's disease (AD). In the present study we attempted to determine the association between *CST3* polymorphism and AD or vascular dementia (VD), and whether such effects are dependent of the *APOE4* allele. The polymorphisms of *CST3* genotype were determined using polymerase chain reactions (PCR) followed by gel electrophoresis in 124 AD, 70 VD, and 115 control individuals. No statistical difference in *CST3B* allele frequencies was observed among all three groups. Associations between *CST3B/B* genotype and AD patients older than 75-year-old, or VD patients younger than 75-year-old were evident. The *APOE4* allele alone significantly increased the odds for the developing AD, but not VD. A logistic regression analysis revealed that either *CST3* or its interaction with *APOE4* were not significant predictors of AD. However, a synergistic association of *CST3* and *APOE4* alleles was observed in predicting VD patients. These results suggest that *CST3* might interact with *APOE4* on conferring vascular pathologies.

Key Words: cystatin C, apolipoprotein E, polymorphism, Alzheimer's disease, vascular dementia

Introduction

Alzheimer's disease (AD) is the most frequent dementia in the elderly, accounting for more than half of all cases (11, 16). In the last decade, emerging evidence suggests the association of genetic risk factors and AD susceptibility. Previous genetic linkage analyses have demonstrated that familial AD is associated with specific mutations to the amyloid-β precursor protein, presenilin 1 and 2 proteins genes (9). In addition, the ε 4 allele of the apolipoprotein E (*APOE*) gene is recognized as a major genetic risk factor for the development of sporadic AD (20). However, despite the clear significance of *APOE4* allele to risk of AD development, only about 30% of patients carry this allele, suggesting the involvement of additional factors in the manifestation of this dementia.

Vascular dementia (VD) is another commonly observed cognitive dysfunction of elderly individuals. Vascular dementia features a dementia syndrome with vascular lesions, white matter changes, hippocampal neuronal loss and some other clinical symptoms also found in AD (4, 23). Interestingly, *APOE4* has also been shown to be associated with a greater risk for VD development (22). Clinically, AD and VD have been

Corresponding author: Dr. Yu-Min Kuo, Department of Cell Biology and Anatomy, National Cheng Kung University College of Medicine, 1 University Rd, Tainan 70101, Taiwan. Tel: +886-6-235-3535 ext 5294, Fax: +886-6-209-3007, E-mail:kuoym@mail.ncku.edu.tw
Received: March 25, 2003; Revised: April 25, 2003; Accepted: April 30, 2003.

treated as distinct diseases. However, there is ample evidence that recognized vascular health risk factors such as hypertension and atherosclerosis increase the risk for both AD and VD (11, 12). Vascular lesions of the brain are frequently detected in the brains of AD patients leading some researchers to hypothesize that AD is fundamentally a vascular disorder (4, 13, 23). This apparent association between AD development and vascular disease risk factors led us to examine the role of cystatin C (*CST3*) gene polymorphisms on probability of dementia development.

Cystatin C is a lysosomal cysteine protease inhibitor involved in neuronal aging or cell death in AD (5, 24). Cystatin C positive neurons were abundantly detected in the cerebral cortices of AD patients, and its regional distribution strongly correlates with pyramidal neurons in cortical layers III and V, the areas of susceptible neurons in AD brains (5, 24). Cystatin C can be amyloidogenic. Ghiso et al. showed that a leucine to glutamine mutation at position 68 of the cystatin C gene resulted in cortical blood vessel amyloid deposits in hereditary cerebral hemorrhage with amyloidosis of the Icelandic type (HCHWA-I) patients (7). In addition, cystatin C was found to co-localize with β-amyloid in the arteriolar walls of patients with AD and patients with cerebral amyloid angiopathy (CAA) (14). Cystatin C is considered a CAA marker because abnormally low levels in cerebrospinal fluid (CSF) were found in both CAA and a late onset sporadic form of CAA with progressive dementia (SCAA) (21).

A polymorphism of *CST3* gene located at position 73 of exon 1 results from an alanine to threonine substitution, which are classified as *CST3A* and *CST3B*, respectively (6). The association of *CST3* polymorphisms with increased risk for sporadic AD has been reported in two Caucasian studies (3, 6). Both studies found that the risk conferred by *APOE4* decreased with age, while the cystatin C polymorphisms conferred risk for AD increased for older subjects. The authors have suggested cystatin C polymorphisms as a good AD risk predictor at ages when *APOE4* is no longer a major risk factor (3, 6). The association between cystatin C and AD development has not yet documented in any Taiwan Chinese population. In addition, there is no data available regarding the relationship between cystatin C genetic variations and risk for VD. In this study, we evaluated a Taiwan Chinese cohort the association of *CST3* polymorphisms with AD or VD development.

Materials and Methods

Patients

The study included 124 patients diagnosed with AD (age range: 60-96, mean ± SD: 76.5 ± 7.1), 70 with VD (range: 60-92, mean ± SD: 74.7 ± 7.5) and 115 ND controls (range: 60-91, mean ± SD: 73.7 ± 6.8). The subjects were Psychiatric and Neurological Outpatient Clinic patients, Taichung Veterans General Hospital, Taiwan. All procedures were approved by the local Institutional Human Care and Use Committee. Informed consent was obtained from all patients or their primary caregivers. Volunteers with no previous history of neurological disease affecting cognition and daily activities, normal bodily condition in physical examinations, and scoring 28 or higher on the Chinese versions Mini-Mental State Examinations (MMSE-T1) served as non-demented controls (ND). The clinical diagnosis of AD was performed according to the NINCDS-ADRDA criteria with minor modifications. Patients were first subjected to the Chinese Version of MMSE, and a score of less than 24 for those cases with education higher than 2nd grade, or 16 for those cases with their highest level of education was below 2nd grade of elementary school was considered to reveal possible dementia. The possible AD patients were subsequently subjected to Clinical Dementia Rating for the final confirmation of the diagnosis (18). The diagnosis of vescular dementia (VD) was compatible with the diagnostic criteria of DSM-IV.

Genotyping of the CST3 and APOE

Genomic DNA was extracted from whole blood samples and used as a template to amplify *CST3* gene with the polymerase chain reactions (PCR). *CST3* genotyping was performed as previously described (6). Briefly, *CST3* gene was amplified using 5'-TGGGAGGGACGAGGCGTTCC-3' and 5'-TCCATGGGGCCCACCAG-3' as forward and reverse primers, respectively. The PCR products (318bp) were digested by restriction enzyme *Sac*II, and resolved by polyacrylalmide gel electrophoresis. For haplotype A, 3 fragments with 41, 226 and 51 bp in size were observed, while for haplotype B, 127 and 191 bp. *APOE* genotyping was performed according to the method of Hixson and Vernier (10). *APOE* gene was PCR amplified using 5'-TCCAAGGACCTGCAGGCGGCGCA-3' and 5'-ACAGAATTCGCCCCGGCCTGGTACACTGCCA as forward and reverse primers, respectively, and the PCR products were digested with the restriction enzyme HhaI. The resulting DNA fragments were resolved by electrophoresis in a 5% LE agarose gel.

Statistical Analysis

Chi-square test was used to compare the *CST3* and *APOE* genotype and allele frequencies between

groups. The odds ratio and its associated 95% confidence intervals (CI) were used to measure the association between the polymorphism of *CST3* and *APOE* and AD or VD. Where appropriate, continuity correction for the odds ratio was applied to account for sparse data. Logistic regression was performed to examine the joint effects of *CST3* and *APOE* genes. The level of statistical significance was set at $P < 0.05$.

Results

The genotype distributions of the *CST3* and *APOE* polymorphisms were in Hardy-Weinberg equilibrium for AD, VD and ND control groups. The CST3B allele frequencies were 11.7%, 13.6% and 11.3% for AD, VD and ND groups, respectively (Table 1). No statistical difference in *CST3B* allele frequencies was observed between AD and ND groups ($\chi^2 = 0.02$, $P = 0.89$), or between VD and ND groups ($\chi^2 = 0.42$, $P = 0.52$). When comparing the homologous genotype frequencies between groups, a higher *B/B* genotype frequency was noticed in the VD patients (5.7%) than ND groups (0.87%) ($\chi^2 = 3.88$, $P < 0.05$). Although not significant, AD patients (4.8%) showed a higher incidence rate trend for *B/B* homozygosity than ND subjects (0.87%) ($\chi^2 = 0.33$, $P = 0.07$).

Since age is a risk factor for AD, we also examined whether there was any change in *CST3B/B* distribution in different age groups. As shown in Table 2, the older age (>75 years) group tended to have higher prevalence of *CST3B/B* homozygotes in AD patients (6.6%) than the ND subjects (0%) (OR = 7.6, 95% CI ranged from 0.4 to 140.9), while no such effect was observed when compared those cases younger than 75 years (2.1% vs. 1.5%, OR = 1.38, 95% CI: 0.1 to 13.7). Interestingly, contrary results were found in the VD group. The VD younger age individuals had a higher incidence rate of *CST3B/B* homozygotes than those of the ND controls (11.4% vs. 1.5%, OR = 6.4, 95% CI: 0.9 to 41.6). No single VD or ND subject with age older than 75 years had a *CST3B/B* genotype (Table 2).

Table 3 shows the joint distribution of *CST3* and *APOE4* polymorphisms by AD, VD and ND groups. As expected, the allele frequency of *APOE4* was significantly higher in AD patients than ND subjects ($\chi^2 = 11.56$, $P < 0.001$) with an OR of 3.1. In contrast, the *APOE4* allele frequency was not significantly different between VD and ND groups ($\chi^2 = 0.59$, $P = 0.44$, OR = 1.4). A logistic regression analysis revealed that both *CST3B* and its interaction with *APOE4* were not significant predictors of AD. However, the interaction was marginally significant ($P = 0.06$) in a logistic regression analysis of *CST3B* and *APOE4* polymorphism in predicting VD patients. The main effects, *CST3B* ($P = 0.14$) and *APOE4* ($P = 0.09$), were not statistically significant.

Table 1. Cystatin C genotypes and allele frequencies in Alzheimer's disease (AD), vascular dementia (VD), and non-demented (ND) control subjects.

	AD (n = 124)	VD (n = 70)	ND (n = 115)
Genotype			
AA	101 (81.5%)	55 (78.6%)	90 (78.3%)
AB	17 (13.7%)	11 (15.7%)	24 (20.9%)
BB	6 (4.8%)	4 (5.7%)	1 (0.87%)
Allele Frequency			
A	219 (88.3%)	121 (86.4%)	204 (88.7%)
B	29 (11.7%)	19 (13.6%)	26 (11.3%)

Discussion

Previously, Finckh et al. reported that AD patients had higher prevalence rates of the *CST3B/B* genotype and *B* allele than those of controls (6). However, we merely observed a trend of elevated *B/B* genotype prevalence in AD subjects, while the incidence rates of *B* alleles and *A/B* genotype were not different between AD and ND individuals. These results imply an association of *CST3B/B* homozygotes, but not *A/B* heterozygotes, to risk for late-onset AD. A recent study using similar approaches reported no association between *CST3B* and AD in Japanese patients (17). These discrepancies may result from the ethnic and environmental differences between these sampled populations. Further studies with larger sample sizes are needed to confirm these observations. Our data did not support the finding by Crawford et al. which, on the contrary, indicated the *CST3G/G* (haplotype *G* is identical to haplotype *A* in this report) as a genetic risk factor for AD in patients 80 years of age or older (3). Interestingly, another independent study from a Spanish Mediterranean population revealed the *CST3B* allele as a risk factor for early-onset AD (2). It seems that the presence of the *CST3B* allele alone is of insufficient statistical power to differentiate AD from ND. Other confounding factors such as inflammations may obscure the connection between *CST3B* allele and AD.

The *CST3* B/B genotype increased the odds for the development of VD in the Taiwan Chinese, even though the proportion of B allele frequency in the VD group is almost identical to that of the ND group, suggesting that homozygous *CST3* B genotype is associated with VD development. Intriguingly, VD patients with the *CST3* B/B genotype were found only

Table 2. *CST3B/B* genotype frequencies in young (< 75 years) and old (≥ 75 years) age groups in Alzheimer's disease (AD), vascular dementia (VD), and non-demented (ND) control groups.

Age	< 75 years			≥ 75 years		
Genotypes	AD (n = 48)	VD (n=35)	ND (n=66)	AD (n = 76)	VD (n=35)	ND (n=49)
BB	1 (2.1%)	4 (11.4%)	1 (1.5%)	5 (6.6%)	0	0
AA/AB	47 (97.9%)	31 (88.6%)	65 (98.5%)	71 (93.4%)	35 (100%)	49 (100%)

Table 3. *CST3B* and *APOE4* allele frequencies in Alzheimer's disease (AD), vascular dementia (VD), and non-demented (ND) control individuals.

		AD	VD	ND
$CST3B^+/APOE4^+$		3	4	0
$CST3B^+/APOE4^-$		20	11	25
$CST3B^-/APOE4^+$		36	8	15
$CST3B^-/APOE4^-$		65	47	75
	$APOE4^+$	39	12	15
	$APOE4^-$	85	58	100
	$CST3B^+$	23	15	25
	$CST3B^-$	101	55	90

in the under 75-year age group. Previous epidemiological study showed that the mortality rate in VD patients was higher than AD patients (15). VD patients with *CST3* B/B genotype might die at earlier ages than non-*CST3* B/B genotype carriers, and this could account for the absence of *CST3* B/B homozygotes in VD patients at 75 or older ages in our study.

In spite of the lower prevalence of *APOE4* alleles in Taiwan Chinese than Caucasian populations, the frequency of *APOE4* allele in AD patients remained significantly higher than the ND control group. The report by Finckh et al. argued against an association between the *CST3B/B* genotype and *APOE4* allele (6). However, we failed to observe this kind of association in our dataset due to the lack of any subject carrying both *CST3B/B* homozygote and *APOE4* allele. Neither did we observe a synergistic effect between the *CST3B* and *APOE4* alleles on AD development risk.

In contrast to previous reports for Caucasians, our Taiwan Chinese VD populations failed to show an overrepresentation of the *APOE4* polymorphic alleles when compared with the ND group (19, 22). Most important, although neither the *APOE4* allele alone nor the *CST3B* allele alone had significant effect on increasing risk for developing VD, a synergistic association of *APOE4* and *CST3B* alleles in the risk of VD for Taiwan Chinese is evident. Furthermore, our results suggested an association between *CST3B/B* genotype and VD development at age younger than 75 years. This is the first linkage of *CST3* polymorphisms to VD pathogenesis has been reported. Cystatin C is well documented to be involved in vascular disorders like CAA, SCAA and hereditary cystatin C amyloid angiopathy (1, 7, 21). In addition, CAA strongly correlated with the presence of cerebral plaques in AD patients (8). However, a physiological manifestation of *CST3* polymorphisms has not yet been documented. Cystatin C generally exists intracellularly in the form of monomers or inactive dimmers, with the latter converting to active monomers before secretion (1). It is possible that the polymorphism located in the signal peptide with an alanine to threonine substitution could change cystatin C aggregation or accumulation, or alter complex formation with other molecules such as β-amyloid peptide, and promote disease development. In addition, the mechanisms underlying the association between *CST3B* and *APOE4* alleles in dementia pathology remain to be explored. More studies using cohorts from other populations will be required to confirm this synergistic effect and may lead to better understanding common pathogenesis mechanisms underlying the recognized forms of dementia.

Acknowledgments

This work is supported in part by grants from National Science Council (NSC90-2320-B-006-072), and Taichung Veterans General Hospital (TCVGH 894001B).

References

1. Benedikz, E., Merz, G.S., Schwenk, V., Johansen, T.E., Wisniewski, H.M. and Rushbrook, J.I. Cellular processing of the amyloidogenic cystatin C variant of hereditary cerebral hemorrhage with amyloidosis, Icelandic type. *Amyloid* 6: 172-182, 1999.
2. Beyer, K., Lao, J.I., Gomez, M., Riutort, N., Latorre, P., Mate, J.L. and Ariza, A. Alzheimer's disease and the cystatin C gene polymorphism: an association study. *Neurosci. Lett.* 315: 17-20, 2001.
3. Crawford, F.C., Freeman, M.J., Schinka, J.A., Abdullah, L.I., Gold,

M., Hartman, R., Krivian, K., Morris, M.D., Richards, D., Duara, R., Anand, R. and Mullan, M.J. A polymorphism in the cystatin C gene is a novel risk factor for late-onset Alzheimer's disease. *Neurology* 55: 763-768, 2000.

4. de la Torre, J.C. Alzheimer disease as a vascular disorder: nosological evidence. *Stroke* 33: 1152-1162, 2002.
5. Deng, A., Irizarry, M.C., Nitsch, R.M., Growdon, J.H. and Rebeck, G.W. Elevation of cystatin C in susceptible neurons in Alzheimer's disease. *Am. J. Pathol*. 159: 1061-1068, 2001.
6. Finckh, U., von der Kammer, H., Velden, J., Michel, T., Andresen, B., Deng, A., Zhang, J., Muller-Thomsen, T., Zuchowski, K., Menzer, G., Mann, U., Papassotiropoulos, A., Heun, R., Zurdel, J., Holst, F., Benussi, L., Stoppe, G., Reiss, J., Miserez, A.R., Staehelin, H.B., Rebeck, G.W., Hyman, B.T., Binetti, G., Hock, C., Growdon, J.H. and Nitsch, R.M. Genetic association of a cystatin C gene polymorphism with late-onset Alzheimer disease. *Arch. Neurol*. 57: 1579-1583, 2000.
7. Ghiso, J., Jensson, O. and Frangione, B. Amyloid fibrils in hereditary cerebral hemorrhage with amyloidosis of Icelandic type is a variant of gamma-trace basic protein (cystatin C). *Proc. Natl. Acad. Sci. USA* 83: 2974-2978, 1986.
8. Haan, J., Maat-Schieman, M.L. and Roos, R.A. Clinical aspects of cerebral amyloid angiopathy. *Dementia* 5: 210-213, 1994.
9. Hardy, J. Amyloid, the presenilins and Alzheimer's disease. *Trends Neurosci*. 20: 154-159, 1997.
10. Hixson, J.E. and Vernier, D.T. Restriction isotyping of human apolipoprotein E by gene amplification and cleavage with HhaI. *J. Lipid Res*. 31: 545-548, 1990.
11. Hofman, A., Ott, A., Breteler, M.M., Bots, M.L., Slooter, A.J., van Harskamp, F., van Duijn, C.N., Van Broeckhoven, C. and Grobbee, D.E. Atherosclerosis, apolipoprotein E, and prevalence of dementia and Alzheimer's disease in the Rotterdam Study. *Lancet* 349: 151-154, 1997.
12. Kivipelto, M., Helkala, E.L., Laakso, M.P., Hanninen, T., Hallikainen, M., Alhainen, K., Soininen, H., Tuomilehto, J. and Nissinen, A. Midlife vascular risk factors and Alzheimer's disease in later life: longitudinal, population based study. *Br. Med*. J. 322: 1447-1451, 2001.
13. Launer, L.J. Demonstrating the case that AD is a vascular disease: epidemiologic evidence. *Ageing Res. Rev*. 1: 61-77, 2002.
14. Levy, E., Sastre, M., Kumar, A., Gallo, G., Piccardo, P., Ghetti, B. and Tagliavini, F. Codeposition of cystatin C with amyloid-beta protein in the brain of Alzheimer disease patients. *J. Neuropathol. Exp. Neurol*. 60: 94-104, 2001.
15. Leys, D., Pasquier, F. and Parnetti, L. Epidemiology of vascular dementia. *Haemostasis* 28: 134-150, 1998.
16. Liu, H.C., Lin, K.N., Teng, E.L., Wang, S.J., Fuh, J.L., Guo, N.W., Chou, P., Hu, H.H. and Chiang, B.N. Prevalence and subtypes of dementia in Taiwan: a community survey of 5297 individuals. *J. Am. Geriatr. Soc*. 43: 144-149, 1995.
17. Maruyama, H., Izumi, Y., Oda, M., Torii, T., Morino, H., Toji, H., Sasaki, K., Terasawa, H., Nakamura, S. and Kawakami, H. Lack of an association between cystatin C gene polymorphisms in Japanese patients with Alzheimer's disease. *Neurology* 57: 337-339, 2001.
18. Morris, J.C. Clinical dementia rating: a reliable and valid diagnostic and staging measure for dementia of the Alzheimer type. *Int. Psychogeriatr*. 9 *(Suppl. 1)*: 173-176, 1997.
19. Myers, R.H., Schaefer, E.J., Wilson, P.W., D'Agostino, R., Ordovas, J.M., Espino, A., Au, R., White, R.F., Knoefel, J.E., Cobb, J.L., McNulty, K.A., Beiser, A. and Wolf, P.A. Apolipoprotein E epsilon4 association with dementia in a population-based study: The Framingham study. *Neurology* 46: 673-677, 1996.
20. Roses, A.D. Apolipoprotein E alleles as risk factors in Alzheimer's disease. *Annu. Rev. Med*. 47: 387-400, 1996.
21. Shimode, K., Kobayashi, S., Imaoka, K., Umegae, N. and Nagai, A. Leukoencephalopathy-related cerebral amyloid angiopathy with cystatin C deposition. *Stroke* 27: 1417-1419, 1996.
22. Slooter, A.J., Tang, M.X., van Duijn, C.M., Stern, Y., Ott, A., Bell, K., Breteler, M.M., Van Broeckhoven, C., Tatemichi, T.K., Tycko, B., Hofman, A. and Mayeux, R. Apolipoprotein E epsilon4 and the risk of dementia with stroke. A population-based investigation. *JAMA* 277: 818-821, 1997.
23. Wallin, A. The overlap between Alzheimer's disease and vascular dementia: the role of white matter changes. *Dement Geriatr. Cogn. Disord*. 9: 30-35, 1998.
24. Yasuhara, O., Hanai, K., Ohkubo, I., Sasaki, M., McGeer, P.L. and Kimura, H. Expression of cystatin C in rat, monkey and human brains. *Brain Res*. 628: 85-92, 1993.