Hum Genet (1997) 101 : 126–129

ORIGINAL INVESTIGATION

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Association of transferrin C2 allele with late-onset Alzheimer's disease

Received: 28 May 1997 / Accepted: 7 August 1997

Abstract Transferrin (Tf), an iron-transporting protein, has many variants, but C1 and C2 variants account for the majority of the population in all races. Since Tf is reported to be immunocytochemically detectable in senile plaques in Alzheimer's disease (AD), we have examined the Tf allele frequency among AD patients. The C2 allele frequency in late-onset AD patients is significantly higher than that in age-matched controls. Unexpectedly, the C2 allele frequency in AD patients homozygous for the ApoE ε4 allele is markedly increased, i.e., it is twice as high as that in the remaining AD patients carrying zero or one copy of the ε4 allele.

Introduction

In addition to apolipoprotein (Apo) E (Corder et al. 1993), specific alleles of α1-antichymotrypsin (Kamboh et al. 1995; Morgan et al. 1997), very low density lipoprotein (VLDL) receptor (Okuizumi et al. 1995), and presenilin 1 (Wragg et al. 1996) have been reported to be significantly associated with Alzheimer's disease (AD), although the first and second may hold true only in restricted populations (Haines et al. 1996; Okuizumi et al. 1996; Chung et al. 1996). The number of genetic risk factors will probably rapidly increase, because the clinical diagnostic accuracy of AD is now at a satisfactory level, because many proteins have now been identified as constituents of the two major lesions of AD, viz., senile plaques and neurofibrillary tangles, and finally because a particular polymorphism of a certain protein of interest can be readily analyzed in blood samples by the polymerase chain reaction (PCR). Indeed, ApoE is known to be associated with the two major lesions (Namba et al. 1991). Thus, if a gene encoding a particular protein that composes the lesions has a certain polymorphism, it could be related to a genetic risk factor that predisposes subjects to AD.

Transferrin (Tf), an iron-transporting protein, has many variants, but the C1 and C2 variants account for the majority of the population of all races (Kamboh and Ferrell 1987). Since Tf has been detected in senile plaques in AD brain (Connor et al. 1992) and administration of sodium ferrous citrate to AD patients is sometimes an effective treatment (Imagawa et al. 1992), we have determined whether the Tf genotype is related to the development of AD. In addition, we have estimated the C2 allele frequency in AD patients with various ApoE genotypes.

Materials and methods

Blood samples were collected from AD patients (n = 201) attending the Hyogo Prefectural Amagasaki Hospital in the Osaka area, from those (n = 93) attending the Nippon Medical College Hospital in the Tokyo area, and from age-matched controls in the Osaka area (n = 291; mean age = 70.9 ± 7.3 years, range = 60–93 years). The C2 allele frequencies were reported to be 0.227–0.241 in the Osaka area and 0.221 in the Tokyo area, respectively (Nakanaga et al. 1991). Thus, there were no significant difference in the C2 allele frequencies between the two areas. Of the 294 AD patients, 97 were diagnosed as having early-onset AD (mean age at onset = 56.5 ± 5.6 years, range = 38–64 years) and 197 as having late-onset AD (mean age at onset = 74.9 ± 6.1 years, range = 65–93 years). The clinical diagnosis of AD was made according to the NINCDS-ADRDA criteria (McKhann et al. 1984).

The Tf polymorphism was determined initially by isoelectric focusing (Kishi et al. 1990) and later by restriction enzymatic cleavage of the PCR-amplified Tf gene (Namekata et al. 1997), as described briefly below. For this procedure, the forward primer was 5′-GCTGTGCCTTGATGGTACCAGGTAA-3′ and the antisense primer was 5′-GGACGCAAGCTTCCTTATCT-3′. The Tf gene was amplified in 35 cycles from genomic DNA by using the primer pairs under the following conditions: denaturation at 94°C

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for 1 min, annealing at 50° C for 2 min, and extension at 72° C for 2 min. *Bst*EII digestion of the PCR product generated an 89-bp fragment from the C1 allele and an intact 110-bp fragment from the C2 allele (Namekata et al. 1997). ApoE genotyping was performed by *Hha*I digestion of the PCR products, as described previously (Hixon et al. 1990). Allelic and genotypic distributions were analyzed by the χ^2 test.

Results

The frequencies of ApoE alleles ε2, ε3, and ε4 in both AD patients and age-matched controls were consistent with those previously reported (Ueki et al. 1993; Dai et al. 1994; see also Table 3). Thus, we can assume that our patients are representative of the Japanese population.

The Tf allele frequency among our age-matched controls agreed well with those in the Osaka and Tokyo areas (Nakanaga et al. 1991; Table 1). AD patients were found to have a higher frequency of the C2 allele than the age-matched controls (control vs AD = 0.22 vs 0.28; $P < 0.04$; Table 1), there being a larger number of C1/C2 heterozygotes in AD patients, compared with age-matched controls (control vs AD = 93/291 vs 126/294). When AD in this series was categorized into its early-onset and late-onset forms, the odds ratio (OR) was significantly increased in late-onset AD patients who had the C2 allele [OR; 1.7, 95% CI (confidence interval); 1.2–2.6, $P < 0.005$; Table 2]. In contrast, no significant increase of the C2 allele frequency was observed in early-onset AD patients.

Table 1 Tf genotypes and allele frequencies

	Age-matched controls (n = 291)		AD patients (n = 294)	
	%	(n)	%	(n)
Tf genotype				
C1/C1	61.8	(180)	51.0	(150)
C1/C2	32.0	(93)	42.9	(126)
C2/C2	6.2	(18)	6.1	(18)
Tf allele frequencies				
C1	0.78		0.72	
C2*	0.22		0.28	

* Statistically significant between control and AD χ^2, $P < 0.04$)

Table 2 Odds ratios (OR) for interaction between the Tf C2 allele and age at onset

	C2 allele copy number	Control n (frequency)	AD patient n (frequency)	OR* 95% CI, P (χ^2)
Early-onset	0	40 (0.62)	55 (0.57)	1.2
	1 or 2	25 (0.38)	42 (0.43)	0.5–2.9, $P < 0.50$
Late-onset	0	140 (0.62)	95 (0.48)	1.7
	1 or 2	86 (0.38)	102 (0.52)	1.2–2.6, $P < 0.005$

* Reference is zero copy of C2 allele

Table 3 Tf C2 allele frequency as related to ApoE ε4 allele

	ApoE ε4		
	–/–	–/+	+/+
AD	0.25 (n = 167)	0.25 (n = 99)	0.50* (n = 28)
Control	0.21 (n = 241)	0.27 (n = 50)	– (n = 0)

* Statistically significant between ε4 +/+ and –/– (χ^2, $P < 0.0003$), and between ε4 +/+ and –/+ (χ^2, $P < 0.0004$)

It is well established that the ε4 allele is a major risk factor for sporadic AD and both early-onset and late-onset familial AD. Our results confirm this as the ε4 frequency is significantly higher in the AD group than in the control group (control vs AD = 0.086 vs 0.264; $P < 0.0001$), as reported previously (Corder et al. 1993; Ueki et al. 1993; Dai et al. 1994). In the present series, none of the controls were homozygous for ε4. We sought to correlate the C2 allele frequency with the copy number of ApoE ε4 allele in all AD patients (Table 3). When subjects had zero or one copy of the ε4 allele, the C2 allele frequency in AD patients was not significantly higher than controls (control vs AD = 0.21 vs 0.25, or 0.27 vs 0.25, respectively). However, the C2 allele frequency in AD patients homozygous for ε4 was as high as 0.5, which was significantly higher than that in AD patients who had a zero copy ($P < 0.0003$) or one copy ($P < 0.0004$) of ε4 allele (Table 3). This higher frequency of C2 allele among ε4-homozygous AD patients was observed in both early-onset AD (0.45) and late-onset AD (0.53). This indicates that the C2 allele is tightly associated with ε4-homozygous AD patients, although Tf and ApoE genes are located on different chromosomes, viz., chromosomes 3 and 19, respectively.

Discussion

Our data suggest that the C2 allele is associated with AD. Its association is significant in late-onset AD, but not in early-onset AD. Although further work is required, we assume that an association of the C2 allele with late-onset AD can be found in other races, because the C2 allele frequencies are similar across several populations, unlike the alleles of the VLDL receptor gene (Okuizumi et al. 1996; Chung et al. 1996). Indeed, based on a small number of cases, the C2 allele frequency in South African AD patients has shown to be higher than that in controls (Van Rensburg et al. 1993).

Several reports suggest that iron and its binding proteins are involved in AD pathogenesis. First, ionic iron, ionic zinc, and aluminum induce the formation of potentially toxic Aβ aggregates in vitro (Mantyh et al. 1993). Second, iron levels affect the processing of amyloid precursor protein to Aβ, suggesting that iron controls α-secretase activity (Bodovitz et al. 1995). Third, the AD brain contains higher concentrations of iron, 67% higher in gray

matter and 27% higher in white matter, than control brain (Ehmann et al. 1986). Numerous ferritin-containing activated microglia are associated with mature plaques, whereas homogeneous diffuse (apparently extracellular) Tf immunostaining around core plaques can be observed, suggesting that Tf may have emigrated from blood vessels. Finally, the level of p97 (melanotransferrin), an iron-binding protein having a high degree of homology to Tf, is remarkably elevated in sera and cerebrospinal fluid from AD patients (Kennard et al. 1996).

Currently, we do not know why and how the C2 allele acts as a risk factor for late-onset AD. The total iron-binding capacity (TIBC) is reported to differ among subjects with different Tf subtypes; the TIBC in C1/C1 subjects is significantly higher than that in C1/C2 subjects, which in turn is higher than that in C2/C2 subjects (Wong and Saha 1986). In this respect, it is interesting to note that fewer Tf-positive oligodendrocytes are present in white matter of the AD brain than in that of control brain. This suggests that, although the AD brain has a higher iron content, the mobilization and thus utilization of iron may be substantially disturbed in the AD brain. A slight but significant decrease in the iron-transporting capacity of serum may be related to the aging process and thus susceptibility to AD.

In AD patients homozygous for ε4, the C2 allele frequency is twice as high as that in the remaining AD groups with zero or one copy of ε4. In contrast, small numbers of C1/C1 homozygotes were found in the AD patients homozygous for ε4 (6/28). Two possible explanations can be proposed for this unusual observation. First, the C2 allele may markedly predispose ε4 homozygotes to AD, although we have been unable to perform statistical analysis to test this, because of the absence of ε4 homozygotes in our age-matched controls. Second, C1/C1 homozygosity could interfere with ε4/ε4 homozygosity; perhaps such an individual dies before birth for unknown reasons. Indeed, one report in the literature suggests that a particular combination of distinct polymorphisms in the two genes increases the risk of spontaneous abortion (Wennberg et al. 1996). Similarly, the combination of C1/C1 and ε4/ε4 may result in unsuccessful fertilization or greatly increase the risk of spontaneous abortion.

We have provided evidence that the C2 allele increases the risk of late-onset AD and is tightly associated with ε4-homozygous AD patients. Although the underlying mechanisms remain unknown, our data suggest that iron metabolism plays a role in the development of late-onset AD and that ε4-homozygous AD is linked to the C2 allele.

Acknowledgements We are grateful to Dr. K. Tokunaga for valuable advice throughout this work. We also thank Drs. Y. Shibata and M. Yanagisawa for providing blood samples, and Dr. T. Hamada for valuable suggestions on statistical analysis. This research was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, by a Grant-in-Aid for Scientific Research from the Ministry of Health and Welfare, by grants from the Takeda Science Foundation and the Kato Memorial Trust for Nambyo Research, and by CREST (Core Research for Evolutional Science and Technology) of the Japan Science and Technology Corporation (JST).

References

Bodovitz S, Falduto MT, Frail DE, Klein WL (1995) Iron levels modulate α-secretase cleavage of amyloid precursor protein. J Neurochem 64:307–315

Chung H, Roberts CT, Greenberg S, Rebeck GW, Christie R, Wallace R, Jacob HJ, Hyman BT (1996) Lack of association of trinucleotide repeat polymorphisms in the very-low-density lipoprotein receptor gene with Alzheimer's disease. Ann Neurol 39:800–803

Connor JR, Menzies SL, St. Martin SM, Mufson EJ (1992) A histochemical study of iron, transferrin, and ferritin in Alzheimer's diseased brains. J Neurosci Res 31:75–83

Corder EH, Saunders AM, Strittmatter WJ, Schmechel DE, Gaskell PC, Small GW, Roses AD, Haines JL, Pericak-Vance MA (1993) Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. Science 261: 921–923

Dai XY, Nanko S, Hattori M, Fukuda R, Nagata K, Isse K, Ueki A, Kazamatsuri H (1994) Association of apolipoprotein E4 with sporadic Alzheimer's disease is more pronounced in early onset type. Neurosci Lett 175:74–76

Ehmann WD, Markesbery WR, Alauddin M, Hossain TIM, Brubaker EH (1986) Brain trace elements in Alzheimer's disease. Neurotoxicology 7:197–206

Haines JL, Pritchard ML, Saunders AM, Schildkraut JM, Growdon JH, Gaskell PC, Farrer LA, Auerbach SA, Gusella JF, Locke PA, Rosi BL, Yamaoka L, Small GW, Conneally PM, Roses AD, Pericak-Vance MA (1996) No genetic effect of alpha 1-antichymotrypsin in Alzheimer disease. Genomics 33:53–56

Hixon JE, Vernier DT (1990) Restriction isotyping of human apolipoprotein E by gene amplification and cleavage with *Hha*I. J Lipid Res 31:545–548

Imagawa M, Naruse S, Tsuji S, Fujioka A, Yamaguchi H (1992) Coenzyme Q10, iron, and vitamin B6 in genetically-confirmed Alzheimer's disease. Lancet 340:671

Kamboh MI, Ferrell RE (1987) Human transferrin polymorphism. Hum Hered 37:65–81

Kamboh MI, Sanghera DK, Ferrell RE, DeKosky ST (1995) APOE4-associated Alzheimer's disease risk is modified by α1-antichymotrypsin polymorphism. Nat Genet 10:486–488

Kennard ML, Feldman H, Yamada T, Jefferies WA (1996) Serum levels of the iron binding protein p97 are elevated in Alzheimer's disease. Nat Med 2:1230–1235

Kishi K, Ikehara Y, Yasuda T, Mizuta K, Sato W (1990) Transferrin polymorphism detected in human urine using isoelectric focusing followed by immunoblotting. Forensic Sci Int 45:225–230

Mantyh PW, Ghilardi JR, Rogers S, DeMaster E, Allen CJ, Stimson ER, Maggio JE (1993) Aluminum, iron, and zinc ions promote aggregation of physiological concentrations of β-amyloid peptide. J Neurochem 61:1171–1174

McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM (1984) Clinical diagnosis of Alzheimer's disease. Neurology 34:939–944

Morgan K, Morgan L, Carpenter K, Lowe J, Lam L, Cave S, Xuereb J, Wischik C, Harrington C, Kalsheker NA (1997) Microsatellite polymorphism of the α1-antichymotrypsin gene locus associated with sporadic Alzheimer's disease. Hum Genet 99:27–31

Nakanaga M, Yasuda T, Tenjo E, Nadano D, Fujiki N, Kishi K (1991) Transferrin polymorphisms in Japanese population: north-south cline in the distribution of the TF*C2 allele. Hum Biol 63:187–195

Namba Y, Tomonaga M, Kawasaki H, Otomo E, Ikeda K (1991) Apolipoprotein E immunoreactivity in cerebral amyloid deposits and neurofibrillary tangles in Alzheimer's disease and kuru plaque amyloid in Creutzfeldt-Jakob disease. Brain Res 541:163–166

Namekata K, Oyama F, Imagawa M, Ihara Y (1997) Human transferrin (Tf): a single mutation at codon 570 determines Tf C1 or Tf C2 variant. Hum Genet 100 : 457–458

Okuizumi K, Onodera O, Namba Y, Ikeda K, Yamamoto T, Seki K, Ueki A, Nanko S, Tanaka H, Takahashi H, Oyanagi K, Mizusawa H, Kanazawa I, Tsuji S (1995) Genetic association of the very low density lipoprotein (VLDL) receptor gene with sporadic Alzheimer's disease. Nat Genet 11:207–209

Okuizumi K, Onodera O, Seki K, Tanaka H, Namba Y, Ikeda K, Saunders AM, Pericak-Vance MA, Roses AD, Tsuji S (1996) Lack of association of very low density lipoprotein receptor gene polymorphism with Caucasian Alzheimer's disease. Ann Neurol 40:251–254

Ueki A, Kawano M, Namba Y, Kawakami M, Ikeda K (1993) A high frequency of apolipoprotein E4 isoprotein in Japanese patients with late-onset nonfamilial Alzheimer's disease. Neurosci Lett 163:166–168

Van Rensburg SJ, Carstens ME, Potocnik FC, Aucamp AK, Taljaard JJ (1993) Increased frequency of the transferrin C2 subtype in Alzheimer's disease. NeuroReport 4:1269–1271

Wennberg C, Kivela A, Holmgren PA (1996) Placental and germ cell alkaline phosphatase RFLPs and haplotypes associated with spontaneous abortion. Hum Hered 45:272–277

Wong CT, Saha N (1986) Effects of transferrin genetic phenotypes on total iron-binding capacity. Acta Haematol 75:215–218

Wragg M, Hutton M, Talbot C, Alzheimer's Disease Collaborative Group (1996) Genetic association between intronic polymorphism in presenilin-1 gene and late-onset Alzheimer's disease. Lancet 347: 509–512