Association between Bleomycin Hydrolase and Alzheimer's Disease in Caucasians

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A recent study showed modest evidence for an increased frequency of the bleomycin hydrolase (BH) V/V genotype in Alzheimer's disease (AD) patients compared with nondemented controls. To test this hypothesis, we examined this polymorphism in 621 rigorously evaluated patients and 502 control subjects (all caucasian) but were unable to detect an association between BH and AD even after controlling for age, gender, and apolipoprotein E (ApoE) genotype. We conclude that this polymorphism does not account for inherited susceptibility to AD in the populations represented in this sample.

Farrer LA, Abraham CR, Haines JL, Rogaeva EA, Song Y, McGraw WT, Brindle N, Premkumar S, Scott WK, Yamaoka LH, Saunders AM, Roses AD, Auerbach SA, Sorbi S, Duara R, Pericak-Vance MA, St. George-Hyslop PH. Association between bleomycin hydrolase and Alzheimer's disease in caucasians. Ann Neurol 1998;44:808–811

Bleomycin hydrolase (BH) is a cysteine protease from the papain family found in a variety of tissues, including brain.[1] Although its native physiological function is unknown, BH is a candidate for the unknown β secretase which is suspected as one of two proteases involved in the production of the amyloid β (Aβ) proteolytic fragment of the amyloid precursor protein (APP) associated with Alzheimer's disease (AD). BH is encoded by a 12-exon single-copy gene located at 17q11.1–11.2 and has a potentially biologically important polymorphic site resulting from a conserved valine to isoleucine substitution at residue 443 near the C-terminus.[2,3] The resulting genotypes are I/I, I/V, and V/V, corresponding to the isoleucine homozygote, heterozygote, and valine homozygote, respectively. Thus, this polymorphism is a candidate susceptibility locus for AD. Montoya and colleagues[4] recently reported an increased frequency of the V/V genotype among AD cases compared with age-matched controls. To test this hypothesis, we examined this polymorphism in a large group of rigorously evaluated caucasian patients and control subjects but were unable to detect an association between BH and AD.

Methods

Subjects

Blood samples were obtained, with institutional review board approval, from 621 caucasian patients (38% male) identified at the Joseph and Kathleen Bryan Alzheimer's Disease Research Center (ADRC) at Duke University (n = 252), at either the Massachusetts ADRC or the Alzheimer's Disease Center at Boston University (n = 234), or through collaborative genetic studies of AD at the University of Toronto (n = 135). The subjects studied in Toronto were ascertained through memory disorder units at the University of Toronto, the Mount Sinai Medical Center (Miami, FL), and the University of Florence (Italy). A diagnosis of AD was established according to standard consensus clinical criteria.[5] The mean age at onset was 70.4 ± 8.8 years. Samples were also obtained from 502 unrelated cognitively normal individuals (51% male) studied at the Duke University ADRC (n = 233), the Boston AD centers (n = 83), and the University of Toronto (n = 186). A large number of these controls were spouses of the patients. The mean age of the controls at examination was 65.6 ± 13.1 years. Noncaucasians were purposely excluded from the study to guard against false-positive or false-negative associations due to population (ie, genetic) stratification within or between AD case and control groups.

Genotyping

Genotypes of apolipoprotein E (ApoE) were determined as previously described.[6] The genotype of BH was determined using allele-specific oligonucleotide methods. An A→G nucleotide polymorphism in the BH coding sequence gives rise to the substitution of Ile443 with Val443.[2,3] The polymerase chain reaction fragment of BH gene was amplified by using the primers 1455, 5′-GTGGTGGTGGACAGGAAGC-3′, and 1456, 5′-CCATGGAAGGAGGAAAGAGC-3′ in a reaction volume of 10 μl containing 100 ng of genomic DNA, 10 pmol of each primer, 250 μM of deoxynucleotide triphosphates, 1.5 mM of $MgCl_2$, and 0.5 units of *Taq* polymerase for 35 cycles of 94°C for 20 seconds, 55°C for 20 seconds, and 72°C for 20 seconds. The polymerase chain re-

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Received Apr 18, 1998, and in revised form May 27. Accepted for publication May 27, 1998.

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action product was denatured in 100 μl containing 0.4 M of NaOH and 25 mM of EDTA and then slot-blotted to duplicate Hybond-N+ (Amersham, Port Hope, Ontario, Canada) nylon membranes. The allele-specific oligonucleotides 1457, 5′-GAACCCATTATCCTGCCAG-3′(Ile443) and 1458, 5′-GAACCCATTGTCCTGCAAG-3′ (Val443) were end labeled and hybridized at 45°C for 2 hours in hybridization buffer (5× Dehardt's, 5× saline sodium citrate (SSC), 0.5% sodium dodecyl sulfate [SDS]), washed to a final stringency of 2× SSC and 0.1% SDS at 58°C, and then exposed to autoradiographic film.

Statistical Analysis

A Fisher exact test was used to compare BH allele frequencies between patients and controls. The influence of BH genotype, ApoE genotype, age, and gender on the odds of developing AD was assessed using logistic regression procedures.[7] To accommodate the polychotomous classification of BH genotype in the regression analysis, indicator variables were constructed representing the I/V and V/V genotypes. These variables took on the value of 1 if the subject had the corresponding genotype and the value of 0 otherwise. The influence of ApoE genotype was partitioned into two indicator variables: one for ApoE 2/3 and one for ApoE 2/4, 3/4, or 4/4. According to this scheme and similar to the approach of Montoya and colleagues,[4] the I/I and 3/3 genotypes were considered as the referents for BH and ApoE, respectively. Age at onset of AD among cases and age at last examination among controls were assigned to the age variable. The possibility of a biological interaction between BH and ApoE was evaluated by performing the regression analyses separately for carriers and noncarriers of the ApoE ε4 allele. Models were evaluated using the LOGISTIC procedure in SAS.[8]

Results

There were no differences between cases and controls overall or stratified by ApoE ε4 status in either allele frequency or genotype frequency (Table 1). The distributions were also similar between men and women as well as among subjects assigned to two groups using the age of 65 years as a cutoff (data not shown). Logistic regression analysis failed to reveal any statistically significant effect of the I/V or V/V genotype on risk of AD, controlling for age at examination, gender, or ApoE ε4 status (Table 2). The conclusions were the same for the subset of ApoE ε4–negative subjects. Among ApoE ε4 carriers, the V/V genotype was apparently protective (odds ratio [OR] = 0.48, confidence interval [CI] = 0.23–0.99), but this nominal result was only marginally significant ($p = 0.047$).

Discussion

The results of this study offer no evidence for an association between AD and BH in the overall data set. When stratified by ApoE genotype, we observed a protective effect of the V/V genotype relative to the I/I genotype among ApoE ε4 carriers. When this marginally significant finding is adjusted for multiple testing, however, the result is no longer significant. Moreover, the pattern of association is inconsistent with the data of Montoya and colleagues,[4] which indicate an *increased* risk associated with this genotype compared with that of the same referent genotype among ApoE ε4 *noncarriers* only. Thus, our association study does not replicate the results of Montoya and colleagues,[4] who concluded that BH is the first susceptibility locus whose impact on risk is confined to individuals lacking an ApoE ε4 allele.

Our sample was certainly large enough to detect an association of the magnitude reported by Montoya and colleagues.[4] In fact, assuming a significance level (α) of 0.05, a power (1-β) of 0.80, and an exposure frequency of 0.10 in controls (ie, those having the V/V genotype), we have the power to detect an OR of 1.5 in our total sample of 621 AD cases and 502 unrelated controls, which is smaller than the effect previously reported for BH. We also considered the possibility that our failure to demonstrate a positive association between the V/V genotype and AD risk was due to an

Table 1. Bleomycin Hydrolase Allele and Genotype Frequencies

		Bleomycin Hydrolase Allele Frequency (%)		Bleomycin Hydrolase Genotype Frequency (%)		
Group	n	Isoleucine	Valine	I/I	I/V	V/V
Alzheimer's disease	621	69.3	30.7	46.2	46.2	7.6
Controls	502	65.5	34.5	40.8	49.4	9.8
		ApoE ε4$^+$ subjects				
Alzheimer's disease	381	69.0	31.0	45.9	46.2	7.9
Controls	132	62.1	37.9	37.1	50.0	12.9
		ApoE ε4$^-$ subjects				
Alzheimer's disease	233	69.3	30.7	45.9	46.8	7.3
Controls	353	67.3	32.7	42.5	49.6	7.9

ApoE = apolipoprotein E.

Table 2. Odds Ratios for Bleomycin Hydrolase Genotype by Apolipoprotein E (ApoE) ε4 Status[a]

	All Subjects		ApoE ε4$^+$ Subjects		ApoE ε4$^-$ Subjects	
Genotype	OR[b]	95% CI	OR	95% CI	OR	95% CI
I/I	1.00	Referent	1.00	Referent	1.00	Referent
I/V	0.84	(0.63–1.11)	0.72	(0.46–1.14)	0.93	(0.65–1.34)
V/V	0.67	(0.40–1.11)	0.48	(0.23–0.99)	0.90	(0.46–1.78)

[a]All odds ratios (ORs) are adjusted for age at examination and gender.
[b]Adjusted for apolipoprotein E genotype.

CI = confidence interval.

admixture of cases and controls from several geographically distinct sources; however, the results were the same in the individual data sets assembled for this study (data not shown). In the study by Montoya and colleagues,[4] pathologically confirmed cases had a higher frequency of V/V compared with clinically diagnosed cases. Thus, it could be postulated that a substantial rate of misclassification might explain our negative results. Although most of our cases have not reached autopsy, misdiagnosis of AD subjects is an unlikely explanation for our negative results, because the accuracy of the clinical diagnosis is more than 90% at the centers participating in this study.

It is noteworthy that BH genotypes were not in Hardy-Weinberg equilibrium in both the patient and control groups ($p < 0.04$ for each), because there was an excess of I/V heterozygote subjects. Nevertheless, this deviation is not significant after adjustment for multiple comparisons.

Although our results exclude an association between BH and AD in the population groups represented in this sample, they do not exclude the possibility that an AD susceptibility gene is located nearby. The findings reported by Montoya and colleagues[4] may simply be due to an association between the BH V allele and an AD gene of relatively high frequency in their case sample. Alternatively, the disparate results between the two studies could be due to population stratification or differences in sampling cases and controls. The small (5.7%) difference in the V/V genotype frequency between AD cases and controls in the study by Montoya and colleagues[4] suggests that even if real, the absolute risks related to this locus would be small. Furthermore, such a small difference is much more sensitive to even small biases in the sample.

Despite the lack of evidence for an association, BH remains an attractive candidate gene for AD. The Aβ peptide associated with AD is a 40- to 42-amino acid fragment proteolytically derived from a much larger APP. Aβ is released from APP by the action of a β secretase at its N-terminus and a γ secretase at its C-terminus. Inhibition of either of these two, as yet unknown, enzymes could reduce the load of neurotoxic Aβ in the brain. We have purified to homogeneity and sequenced a cysteine proteinase from AD brain based on its ability to cleave a synthetic peptide harboring the β-secretase site.[9] Upon sequencing of several internal peptides and comparison with known sequences, we discovered that this proteinase corresponds to the human homologue of BH (W. T. McGraw and Abraham, submitted). Analysis of the yeast BH crystallographic structure revealed that the C-terminal tail obstructs the primed sites of the active site cleft.[10] Subsequent analysis demonstrated that removal of three C-terminal residues was sufficient to convert BH from an aminopeptidase to an endopeptidase[11] as often occurs in other members of the papain superfamily of proteinases.[12] The isoleucine/valine polymorphism at position 443 in the C-terminus falls in a location that precedes the insertion of the C-terminal tail into the active site cleft. Because this polymorphism could affect the structural stability and/or conformation of the active site-occluding tail, we predicted that one of the isoforms would be more frequent in AD cases compared with controls. A lack of an association suggests that this amino acid change has little effect on the function of the protein, however. Alternatively, in light of positive evidence for an association in another population,[4] the impact of the Ile443/valine substitution on AD risk may be realized only on a specific genetic or environmental background infrequent in our sample. Additional biochemical and molecular analysis of the BH variants should clarify the contribution of this gene to AD susceptibility.

Support for this project came from the following federal grants: AG09029 (L.A.F.), NS31153 (M.A.P.-V., J.L.H.), AG05128 (A.D.R., A.M.S.), and AG0995 (C.R.A.); a LEAD award for excellence in Alzheimer's disease (A.D.R.); grants RG2-96044 (M.A.P.-V.) and RG2-96 (J.L.H.) and a Zenith Award (C.R.A.) from the Alzheimer Association; and grants from the Medical Research Council of Canada, Canadian Genetic Diseases Network, Alzheimer Association of Ontario, Howard Hughes Medical Research Foundation, E.J.L.B. Foundation (P.H. St. G.-H.), and Telethon E352 (S.S.). Dr Rogaeva is a recipient of the Peterborough Burgess Fellowship; Dr McGraw is supported in part by NIH grant T32-

NS07152; and Dr Premkumar is supported by NIH grant T32-AG00115.

We thank the patients with Alzheimer's disease and their families, whose help and participation made this work possible, as well as the clinical and research personnel of the Boston University ADC and Neurological Referral Center, the Joseph and Kathleen Bryan ADRC, the Duke University Medical Center Section of Medical Genetics, and the Massachusetts General Hospital ADRC.

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Somatic and Limbic Cortex Activation in Esophageal Distention: A Functional Magnetic Resonance Imaging Study

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Little is known about the cerebral representations of visceral sensations in humans. Using functional magnetic resonance imaging (fMRI), we mapped the cortical areas of the human brain that were activated by mechanical stimulation of the esophagus in 5 healthy volunteers. Stimulation probes were placed into the distal part of the esophagus and inflated to produce a local distention. The cerebral activation pattern was related to the strength and quality of the stimulus. The weakest stimulus accompanied by a well-localized albeit weak retrosternal sensation activated only the parietal opercular cortices, probably including the secondary somatosensory cortex (SII). Additional activation of the primary sensorimotor cortex (SI) at the level of the face and mouth representation as well as of the right premotor cortex was found during repetitive distention of the esophagus at 0.5 Hz. Repetitive stimulation at 1 Hz additionally activated the insula bilaterally. The strongest distention stimulus, which caused a painful retrosternal sensation, resulted in an activation of the anterior cingulate cortex. Our findings demonstrate that SII is the primary cortical target of visceral afferents originating in the esophagus. Limbic structures become engaged when the visceral sensation is unpleasant or painful.

Binkofski F, Schnitzler A, Enck P, Frieling T, Posse S, Seitz RJ, Freund H-J. Somatic and limbic cortex activation in esophageal distention: a functional magnetic resonance imaging study. Ann Neurol 1998;44:811–815

Knowledge about the cerebral representation of visceral sensation in humans is not conclusive.[1] Recently, evoked potential studies,[2,3] neuromagnetic recordings,[4] and a positron emission tomography study[5] have sug-

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Received Feb 3, 1998, and in revised form Apr 27. Accepted for publication May 29, 1998.

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