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Investigation of the effect of brain-derived neurotrophic factor (BDNF) polymorphisms on the risk of late-onset Alzheimer's disease (AD) and quantitative measures of AD progression

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Abstract

BDNF is a functional candidate gene for AD, owing to its role in neuronal development and survival. The Val66Met (G196A), along with another C270T polymorphism has been associated with AD, however, the effects seem to be inconsistent across studies. We examined the association of the G196A and C270T polymorphisms with sporadic late-onset AD (LOAD) in a large American White cohort of 995 AD cases and 671 controls and an American Black cohort of 64 AD cases and 45 controls. We also examined the association of these polymorphisms with quantitative measures of AD progression, including age at onset (AAO), disease duration and Mini-Mental State Examination (MMSE) scores. No significant difference in allele, genotype or estimated haplotype frequencies was observed between AD cases and controls within the American White and Black cohorts for the G196A and C270T polymorphisms. However, the frequency of the *196*A* allele was significantly lower in American Black subjects compared to Whites. While MMSE scores were significantly lower in C270T/CT carriers compared to C270T/CC subjects only among American Blacks, no such effect was observed among American Whites. The BDNF polymorphisms did not affect AAO or disease duration measures in American Whites or Blacks. Our finding does not support any association between the BDNF/G196A or C270T polymorphism and the risk of sporadic LOAD among American Whites or Blacks. The significant effect of the C270T polymorphism observed on MMSE scores among American Blacks needs to be further explored in a larger cohort.

Keywords: Alzheimer's disease; BDNF; Polymorphism

AD is a debilitating neurodegenerative disorder, resulting in the progressive decline of cognitive functions. Neuritic plaques, neurofibrillary tangles and neuronal cell loss, particularly in the hippocampus and cerebral cortex are neuropathological hallmarks of AD. To date, mutations in three genes, including amyloid precursor protein (APP), presenilin-1 (PSEN1) and presenilin-2 (PSEN2) have been shown to cause early-onset autosomal dominant AD [9]. So far, only the apolipoprotein E (APOE) gene, specifically the *APOE4* allele has been identified as a susceptibility factor for the more complex, sporadic LOAD, which accounts for bulk of all AD cases. However, about 50% of LOAD cases do not possess the *APOE4* allele, and not all individuals carrying the *APOE4* allele develop AD [28]. Twin studies based on incident [25] and prevalent [8] AD cases estimate that genetic factors account for about 48 and 74% of LOAD risk, respectively. Evidence from genetic linkage studies suggests the existence of multiple risk genes for LOAD [14]. Genetic susceptibility to sporadic LOAD cannot be completely attributed to the genes identified so far, and the search for polymorphisms in candidate genes is ongoing.

Brain-derived neurotrophic factor (BDNF) has been implicated as a functional candidate gene for AD, owing to its role in neuronal development, survival and function. BDNF belongs to a subclass of neurotrophic factors called

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neurotrophins, which are secreted peptides that serve as target-derived growth factors for specific neuronal populations [22]. BDNF supports the maintenance of the dopaminergic neurons of the substantia nigra, and the basal forebrain cholinergic neurons which direct projections into the hippocampus and neocortical areas of the brain [22]. Since these are the regions with the most severe neuronal loss in Parkinson's disease (PD) and AD, respectively, BDNF has been implicated as an important factor in the etiology of both PD and AD.

A marked downregulation of BDNF mRNA and protein has been reported in the hippocampus and temporal cortex of autopsied AD brains, suggesting that this decrease may contribute to the neuronal atrophy in AD [3,10,11,26]. Deposits of BDNF-immunoreactive material have been found in senile plaques in AD patients [23]. Dystrophic neurites surrounding senile plaques have been shown to be strongly immunolabeled with BDNF, while a reduced intensity of BDNF immunoreactivity has been observed in both tangle-bearing as well as tangle-free neurons of AD subjects [6].

The BDNF gene has been localized to human chromosome 11p13 [19]. Egan et al. [5] identified a putative functional polymorphism, Val66Met (G196A), in the pro-peptide region of BDNF and found that carriers of the *196*A/Met* allele were associated with poor performance on learning and memory tasks. Considering that learning and memory functions are impaired in AD, the *196*A/Met* allele would more likely be associated with the risk of AD. On the contrary, however, Ventriglia et al. [30] reported a significant association for the more common *196*G/Val* allele with an increased risk for sporadic AD, while other studies in smaller cohorts did not find any significant association between G196A polymorphism and AD [2,29]. Another BDNF/C270T polymorphism located in the 5′UTR, has been shown to be associated with AD with conflicting results [1,15,24,27].

In the present study, we examined the effect of the G196A and C270T polymorphisms on the risk of sporadic LOAD in a large American White cohort of 995 AD cases and 671 controls and an American Black cohort of 64 AD cases and 45 controls. In addition, we sought to determine whether the BDNF polymorphisms had any effect on quantitative measures of AD progression such as AAO, disease duration and MMSE scores. Such an approach has been proposed in mapping complex diseases, whereby examining the effect on quantitative traits could help identify patterns of transitional phenotypes that precede a disease outcome [16].

The LOAD sample comprised of 995 American Whites (66.9% females; mean age at onset of 72.3 ± 6.3 years) and 65 American Blacks (71.9% female; mean age at onset 72.30 ± 5.78 years) from the University of Pittsburgh Alzheimer's Disease Research Center (ADRC). All cases were clinically evaluated and satisfied the NINCDS/ADRDA criteria for probable AD [21]. The control sample comprised of 671 American Whites (61.2% females; mean age 75.7 ± 5.3 years) and 45 American Blacks (mean age 70.49 ± 5.67 years; 73.3% female) recruited from the same Western Pennsylvania region from which the patients were drawn. All controls were found to be cognitively intact following extensive neuropsychiatric evaluation, including medical history, physical examination, neurological history and examination, and neuropsychological assessment [31]. The severity of cognitive impairment was assessed from the MMSE scores measured at baseline and during the last phase of the study [7]. All study protocols were approved by the University of Pittsburgh, Institutional Review Board.

The G196A and C270T genotypes were determined by pyrosequencing on the PSQ^{TM} HS 96 system (Biotage, Uppsala, Sweden). Duplex pyrosequencing assay was performed using the following PCR (F, R) and sequencing (S) primers for G196A: F—5′-GTACTCTGGAGAGCGTGAATGG-3′; R—5′-ACTACTGAGCATCACCCTGGA-3′ (Biotinylated); S—5′-GCTGACACTTTCGAACAC-3′ and C270T: F—5′-CTGGGCGCTGGAGCCAGAATC-3′; R—5′-CTCCTGCACCAAGCCCCATTC-3′ (Biotinylated); S—5′-CCGCCGCCGGGGA-3′. In order to determine each genotype, single-stranded DNA was purified from the 25-μl PCR reaction with streptavidin sepharose (Amersham Biosciences, Piscataway, NJ). The streptavidin sepharose with captured DNA were then sequentially washed with 70% ethanol, followed by 0.2 M NaOH and finally with washing buffer (10 mM Tris–acetate, pH 7.6). The different genotypes were determined in a 96-well reaction plate format by annealing 10 pmol of the corresponding sequence-specific primer to the single-stranded DNA. Annealing was conducted in the annealing buffer by heating the sample to 90 °C and allowing it to cool to room temperature. All pyrosequencing biochemicals were obtained from Biotage (Uppsala, Sweden) and results were analyzed using the PSQ HS 96 SNP software.

Data were analyzed separately for the American White and Black cohorts to control for any differences due to population stratification. The number of samples genotyped for the two polymorphisms differed because the data for the G196A polymorphism included genotypes on 375 subjects screened using a simplex pyrosequencing assay (using the same G196A primers as mentioned above) (Table 1a). Additional differences in numbers were due to failed PCR reactions for the C270T polymorphism. Allele frequencies were calculated by the allele counting method. Hardy-Weinberg equilibrium was tested for each polymorphism using Chi-square test. Pair-wise linkage disequilibrium (LD) between markers was estimated using the D' method [17]. Multiple logistic regression models were used to examine the association of each genetic marker with AD. The estimated odds ratios (ORs) were adjusted for the effect of significant covariates, such as age, sex and APOE. Quantitative traits such as AAO, disease duration and MMSE scores were compared between different genotype groups using one-way analysis of variance and adjusted for the effect of significant covariates such as sex and APOE. The disease duration was estimated by subtracting the age at onset from the age at death for American White AD subjects. The age at death information was not available for the American Black AD subjects. All computa-

Table 1a
Distribution of BDNF polymorphisms in American White LOAD cases and controls

Genotype	AD		Controls		p
	n	%	n	%	
G196A (Val66Met)					
GG	662	66.53	456	67.96	
AG	299	30.05	197	29.36	
AA	34	3.42	18	2.68	
Total	995		671		
Allele frequency					
G/A		81.6/18.4		82.6/17.4	0.46
C270T					
CC	629	87.48	454	86.81	
CT	86	11.96	69	13.19	
TT	4	0.56	0	0.00	
Total	719		523		
Allele frequency					
C/T		93.5/6.5		93.4/6.6	0.92

Table 1b
Distribution of BDNF polymorphisms in American Black LOAD cases and controls

Genotype	AD		Controls		p
	n	%	n	%	
G196A (Val66Met)					
GG	59	92.20	42	93.30	
AG	5	7.80	3	6.70	
AA	0	0.00	0	0.00	
Total	64		45		
Allele frequency					
G/A		96.1/3.9		96.7/3.3	0.81
C270T					
CC	54	93.10	38	90.50	
CT	4	6.90	4	9.50	
TT	0	0.00	0	0.00	
Total	58		42		
Allele frequency					
C/T		96.6/3.4		95.2/4.8	0.63

tions were performed using *R* statistical 1.8.1 program [12]. The two-site haplotype frequencies were estimated using the Expectation-Maximization algorithm in the EH software program (http://linkage.rockefeller.edu/ott/eh.htm). Haplotype frequencies between AD cases and controls were compared using *z* test.

The genotype distributions of both polymorphisms, G196A and C270T followed Hardy-Weinberg equilibrium in both American White and Black AD cases and controls. The *270*T* allele was in significant LD with the *196*G* allele ($D' = 0.757$; $p < 0.0001$) in the American White cohort. Such LD was not apparent among the American Black cohort. The genotype and allele frequencies were comparable between AD cases and controls in the American White cohort (Table 1a). No significant difference in allele or genotype frequencies was observed between American Black AD cases and controls for the G196A or the C270T polymorphisms (Table 1b). Furthermore, no significant differences in allele or genotype frequencies were observed among American Whites or Blacks when the subjects were stratified by their ApoE4 carrier status. However, the frequency of the *196*A* allele was significantly lower in American Black controls compared to White controls (0.039 versus 0.184; $p < 0.0001$) and in American Black AD cases compared to White AD cases (0.033 versus 0.174; $p < 0.0001$).

The estimated haplotype frequencies for the two markers observed in American White and Black subjects are presented in Tables 2a and 2b, respectively. Three (haplotypes 1, 2, and 4) of the four possible haplotypes accounted for all of the chromosomes examined. Haplotype 3 was absent in the subjects examined. The overall haplotype frequencies were not statistically different between LOAD cases and controls for Whites ($p = 0.73$) or Blacks ($p = 0.98$).

We also examined the association of the BDNF polymorphisms with quantitative measures of AD progression, such as AAO, disease duration and MMSE measures scored at baseline (MMSE baseline) and during the last phase of the study (MMSE last). No significant difference was observed between the G196A genotypes among American Whites or Blacks, and between the C270T genotypes among American Whites (Tables 3a and 3b). However, the MMSE scores measured at baseline and during the last phase of the study significantly differed between the C270T genotypes among American Blacks ($p = 0.031$ and $p = 0.005$, respectively).

The aim of our current study was to further examine the contrasting reports for the association of the Val66Met and

Table 2a
Estimated haplotype frequencies among American White LOAD cases and controls

Haplotype	SNPs			Haplotype frequency (%)			p
	G196A (Val66Met)	C270T		Total ($n = 1229$)	AD ($n = 718$)	C ($n = 511$)	
1	A	C		17.9	17.8	18.2	0.80
2	G	C		75.5	75.7	75.2	0.78
3	A	T		0.0	0.0	0.0	–
4	G	T		6.5	6.5	6.6	0.92
			$\ln(L)$	−1391.52	−810.84	−580.04	0.73[a]

[a] Calculated from the T5 statistic: $2[\ln(L)_{case} + \ln(L)_{control} - \ln(L)_{case+control}]$; d.f. = 3.

Table 2b
Estimated haplotype frequencies among American Black LOAD cases and controls

Haplotype	SNPs		Haplotype frequency (%)			p
	G196A (Val66Met)	C270T	Total (n = 99)	AD (n = 57)	C (n = 42)	
1	A	C	3.5	3.5	3.6	0.98
2	G	C	92.4	93.0	91.7	0.73
3	A	T	0.0	0.0	0.0	–
4	G	T	4.0	3.5	4.8	0.66
		ln(L)	−53.09	−28.97	−24.02	0.98[a]

[a] Calculated from the T5 statistic: $2[\ln(L)_{case} + \ln(L)_{control} - \ln(L)_{case+control}]$; d.f. = 3.

Table 3a
Association of BDNF polymorphisms with quantitative traits in American Whites

Trait	GG	AG	AA	p
Val66Met				
Age at onset[a]	72.4 ± 6.1 [613]	72.7 ± 6.0 [276]	70.9 ± 6.7 [34]	0.164
Disease duration[a]	10.1 ± 4.3 [138]	10.5 ± 4.5 [54]	8.7 ± 3.8 [9]	0.784
MMSE baseline[b]	23.2 ± 4.9 [967]	23.0 ± 4.7 [430]	23.7 ± 4.4 [41]	0.798
MMSE last[b]	21.3 ± 5.5 [914]	21.0 ± 5.3 [424]	20.6 ± 5.8 [47]	0.445
Trait	CC	CT	TT	p
C270T				
Age at onset[a]	72.2 ± 5.9 [575]	71.3 ± 5.9 [75]	70.2 ± 5.8 [4]	0.824
Disease duration[a]	10.6 ± 4.4 [162]	10.3 ± 4.9 [20]	11.7 ± 1.2 [2]	0.421
MMSE baseline[b]	23.2 ± 4.7 [900]	23.4 ± 5.3 [124]	23.5 ± 6.8 [4]	0.634
MMSE last[b]	21.2 ± 5.4 [887]	20.8 ± 5.8 [129]	22.5 ± 6.0 [3]	0.706

Multivariate ANOVA performed in cases only and cases and controls.
[a] Adjusted for gender and ApoE4.
[b] Adjusted for $age_{controls}$, age at $onset_{cases}$, disease status, gender and ApoE4. Values in parentheses indicate the number of subjects in each genotype.

the C270T polymorphisms with the risk of AD, using a larger American White cohort of 995 AD cases and 671 controls. We also examined the distribution of these polymorphisms in a smaller American Black cohort of 64 AD cases and 45 controls. To our knowledge, this is the first study examining the distribution of the BDNF polymorphisms among Black AD cases and controls. However, no evidence of significant association was observed between the BDNF polymorphisms and the risk for LOAD in our dataset of American White or Black subjects. We observed a significant difference in allele frequency for the G196A polymorphism between the two ethnic groups, with the *196*A/Met* allele being significantly lower in American Blacks compared to Whites. The frequency of 3.3% for the *196*A/Met* allele seen in American Black controls is the lowest among all ethnic groups studied so far. The frequency of 17.4% for the *196*A/Met* allele seen in our American White controls is comparable to reports in other European Americans (18%) [5]. Studies from Italy have reported frequencies ranging from 23.7 to 29.7% [1,30] for the *196*A/Met* allele, while the highest frequencies so far have been reported among Japanese controls, ranging from 41.1 to 44.5% [13,20]. Thus, there appears to be considerable interethnic differences in the allele frequencies for the BDNF G196A polymorphism among Blacks, Whites and the Japanese cohorts. The significantly low frequency of the *196*A/Met* allele seen in our American Black cohort indicates the possibility of this allele being mainly Caucasian in origin and may represent a partial Caucasian heritage among these American Black subjects. Screening this polymorphism in other African Black populations may help ascertain the frequency of the *196*A/Met* allele among Blacks. Kunugi et al. [15] and Riemenschneider et al. [27] reported a significant risk effect for the *270*T* allele of the C270T polymorphism among Japanese LOAD and German EOAD subjects, respectively. However, we did not detect any association for the C270T polymorphism in our American Black and White LOAD cohort. Allele frequencies for the C270T polymorphism appear to be comparable among different ethnic groups studied so far.

Table 3b
Association of BDNF polymorphisms with quantitative traits in American Blacks

Trait	GG	AG	AA	p
Val66Met				
Age at onset[a]	72.1 ± 5.9 [58]	73.1 ± 6.1 [5]	–	0.344
MMSE baseline[b]	20.4 ± 4.9 [87]	22.0 ± 4.3 [8]	–	0.217
MMSE last[b]	20.2 ± 4.7 [88]	19.8 ± 1.7 [8]	–	0.606
Trait	CC	CT	TT	p
C270T				
Age at onset[a]	72.0 ± 5.8 [53]	71.3 ± 3.9 [4]	–	0.782
MMSE baseline[b]	20.9 ± 4.6 [79]	16.6 ± 5.8 [7]	–	0.031
MMSE last[b]	20.2 ± 4.7 [82]	19.4 ± 5.5 [8]	–	0.005

Multivariate ANOVA performed in cases only and cases and controls.
[a] Adjusted for gender and ApoE4.
[b] Adjusted for $age_{controls}$, age at $onset_{cases}$, disease status, gender and ApoE4. Values in parentheses indicate the number of subjects in each genotype.

Since Egan et al. [5] demonstrated a role for the BDNF/Val66Met polymorphism in human memory and hippocampal function, we examined the effect of the Val66Met (G196A) and C270T polymorphisms on measures of cognitive function such as MMSE scores. However, no significant effect of the BDNF/Val66Met polymorphism was observed on MMSE scores among American Whites or Blacks. The BDNF/C270T polymorphism did not affect MMSE scores among American Whites, however, in contrast we observed significantly lower MMSE scores among CT carriers compared to wildtype CC subjects among American Blacks. However, this data needs to be interpreted with caution due to the small sample size of the American Black cohort. Additional studies using a larger Black cohort need to be performed to confirm this effect of the C270T polymorphism on MMSE scores. Quantitative traits such as AAO of disease have been found to be influenced by genes [18]. The *APOE*4* allele has been shown to decrease AAO in AD patients in a dosage-dependent manner [4]. We also examined the effect of the BDNF polymorphisms on covariates such as AAO and disease duration, however, no significant effect was observed in American Whites or Blacks.

Our results emphasize the importance of replication studies using larger cohorts to differentiate between the true positive associations identified in smaller datasets from spurious findings. Several candidate genes showing initial positive associations have generated negative findings in replications studies due to issues with insufficient power or sample heterogeneity. In the present study, our large American White sample of 995 LOAD cases and 671 controls had 99.99% power to detect a risk effect of 1.994 as previously reported by Ventriglia et al. [30] for the Val66Met (G196A) polymorphism. Similarly, our American White sample had 100% power to detect an odds ratio of 3.8 as reported by Kunugi et al. [15] for the C270T polymorphism. Moreover, all cases and controls within each cohort were from the same geographic region and of the same ethnic origin, thus reducing potential effects of population stratification. Another important issue to be considered is the need for replication of association studies in different ethnic groups. The polymorphism under study could be in LD with the true disease variant, and the extent of LD may vary between different ethnic populations. Therefore, the possibility of the BDNF alleles associated with AD risk in previous studies being in significant LD with the true disease variant within BDNF, or in nearby genes cannot be completely ruled out. About 95 SNPs in the BDNF gene have been listed in the public database, and their comprehensive analyses in future studies may help identify the putative functional SNP in this gene.

In conclusion, although there is evidence from previous studies for the functional effects of the BDNF/Met allele, we did not find any association between the G196A and C270T polymorphisms and the risk of AD in our American White and Black cohorts.

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