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Polymorphisms in the PON gene cluster are associated with Alzheimer disease

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Paraoxonase is an arylesterase enzyme that is expressed in the liver and found in the circulation in association with apoA1 and the high-density lipoprotein, and prevents the accumulation of oxidized lipids in low-density lipoproteins *in vitro*. Common polymorphisms in genes encoding paraoxonase are established risk factors in a variety of vascular disorders including coronary artery disease and carotid artery stenosis, but their association with Alzheimer disease (AD) is controversial. We tested the association of 29 SNPs in PON1, PON2 and PON3 with AD in 730 Caucasian and 467 African American participants of the MIRAGE Study, an ongoing multi-center family-based genetic epidemiology study of AD. Eight SNPs were associated with AD in the African American families ($0.0001 \leq P \leq 0.04$) and two SNPs were associated with AD in Caucasian families ($0.01 \leq P \leq 0.04$). Of note, the pattern of association for the PON1 promoter SNP −161[C/T] was the same in both ethnic groups ($P = 0.006$). Haplotype analysis using sliding windows revealed 11 contiguous SNP combinations spanning the three PON genes with significant global test scores ($0.006 \leq P \leq 0.04$) in the two ethnic groups combined. The most significantly associated haplotype comprised SNPs in the region spanning the −161[C/T] SNP ($P = 0.00009$). Our results demonstrate association between AD and variants in the PON gene cluster in Caucasians and African Americans.

INTRODUCTION

Alzheimer disease (AD) is the most common form of dementia among the elderly, characterized clinically by progressive-onset memory loss and cognitive decline, and neuropathologically by amyloid plaques and neurofibrillary tangles evident in autopsy brains of affected individuals (1,2). In addition to these neuropathological hallmarks, AD brains frequently display vascular pathologies including cerebrovascular amyloid angiopathy (3–5) and atherosclerosis (6–11). Late-onset AD (age of onset >60) does not appear to be transmitted in any Mendelian mode, but has a significant heritable component (12). To date, the only unequivocal genetic risk factor of late-onset AD is APOE (13). Specific variants of APOE are associated with abnormalities in lipid and cholesterol homeostasis leading to premature atherosclerosis as well as AD (13–18). Although the specific mechanisms by which APOE variants affect AD are still debated (19), its involvement in the disease is unequivocal and suggests an involvement of the lipid transport system in the etiology of AD.

The human PON locus encoding paraoxonase spans ~120 kb on chromosome 7q21.3 and contains the three members of the PON gene family (20,21). Paraoxonase is a component of the lipid transport system, is physically associated with apoA-1 and high-density lipoprotein *in vivo* (22–24) and prevents the accumulation of atherogenic oxidized lipids in low density lipoproteins *in vitro* (25,26). Specific coding and regulatory polymorphisms at the PON locus as well as deficient levels of paraoxonase activity measured in sera (27) are associated with a variety of vascular disorders

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resulting from atherosclerosis of blood vessels (28–34). PON1 and PON3 are expressed primarily in liver, whereas PON2 is widely expressed including in brain, heart, kidney, liver, lung, testis and the gastrointestinal tract (35–37). Several recent studies observed association of PON polymorphisms with AD and vascular dementia (38–43), but the interpretation is unclear in light of multiple negative reports (44–47).

The present investigation was designed to comprehensively explore the association between AD and 29 polymorphisms spanning the entire PON locus in two ethnic cohorts, consisting primarily of AD probands and their non-demented sibs. Our results reveal significant evidence of association between polymorphisms in this locus and AD and suggest that paraoxonase may play an important role in the etiology AD.

RESULTS

A total of 730 Caucasian and 467 African American AD cases and non-demented controls (see Table 1 for subject characteristics) were genotyped for 29 SNPs in the PON gene region (Table 2). The linkage disequilibrium (LD) structure (Fig. 1) was similar in the African American and Caucasian groups. Two blocks of high-LD were observed: one encompassing PON1 (SNPs 1–8) and the other encompassing PON3 and PON2 (SNPs 11–28). These two blocks were separated by a region of low-LD spanning 16.3 kb between SNPs 8–11.

The family-based association tests (FBAT; Fig. 2 black bars) and a generalized estimating equation approach adjusting for age and sex in the full data set (GEE; Fig. 2 gray bars) were used to test for association of each individual SNP with AD. Eight SNPs were associated with AD in the full African American sample ($0.0001 \leq P \leq 0.04$) and two SNPs were associated with AD in the Caucasian sibships ($0.01 \leq P \leq 0.04$). The location of significant associations observed within the PON cluster was different in African Americans and Caucasians (Fig. 2). FBAT analysis conducted in the pan-ethnic sibships data set (Fig. 2; upper panel) revealed four SNPs associated with AD ($0.007 \leq P \leq 0.03$). Results for these four SNPs were more significant in the pan-ethnic data set than in either ethnic subgroup, indicating that alleles in these SNPs have the same pattern of association in both ethnic groups. Adjustment for APOE ε4 carrier status did not substantively change these conclusions (data not shown).

Haplotype analysis using sliding windows was performed in the pan-ethnic sibships data set to identify regions with variants most likely contributing directly to the observed association (Fig. 3A). Eleven contiguous SNP combinations with a significant global test score were detected ($0.006 \leq P \leq 0.04$) and further examined using haplotype specific tests. In haplotype specific tests, nine deleterious and seven protective disease associated haplotypes were observed ($0.00009 \leq P \leq 0.04$). The strongest associations were found in the region spanning the PON1 promoter. In SNP subgroup 7–10, haplotype V is common (20% frequency) and has a highly significant predicted deleterious effect, whereas haplotype X in this group appears protective. These two haplotypes differ only at SNP10 and exhibited opposite effects on AD risk (T is deleterious and C is protective). Furthermore, in all deleterious haplotypes encompassing SNP10, a T was found in this position, whereas C was found in all protective haplotypes.

Table 1. Sample characteristics

Ethnic group	Cohort	Number	Mean age	Male (%)
African Americans	Probands	237	76.7	24.0
	Unaffected sibs	143	72.9	32.9
	Affected sibs	6	76.5	0
	Spouses	47	72.2	36.2
	Neighborhood-based	34	72.8	23.5
Caucasians	Probands	265	73.9	33.2
	Unaffected sibs	329	70.3	40.4
	Affected sibs	12	75.6	25.0
	Spouses	124	71.7	58.9

In a further attempt to discern SNPs that impact AD risk, global haplotype tests were performed for all pairwise SNP combinations. As shown in Figure 3B, nearly all combinations involving SNP10 demonstrated significant evidence of association with AD. Taken together with the results for the individual SNPs and sliding window haplotype analyses, this finding suggests that SNP10, or an untested proximate SNP in LD with SNP10, may be a functional determinant of disease susceptibility.

DISCUSSION

In this study, we observed significant evidence of association between polymorphisms in the PON gene cluster and AD in African Americans and Caucasians. Our finding of significant associations in two ethnically distinct samples tempers the concern that these associations may be spurious. Furthermore, our results identify specific genomic regions and haplotypes within the PON locus that may harbor biologically important variant(s). These regions include the proximal promoter and 5′ transcribed region of the PON1 gene as well as portions of the transcribed regions of PON2 and PON3. Our study has a number of advantages over previous genetic association studies of PON with AD including the utilization of a discordant sib pair design that reduces error due to population stratification and analysis of a large number of SNPs to maximize the power to detect association.

Previous studies have identified regulatory and coding PON1 variants that are associated with low PON1 mRNA transcript levels, low paraoxonase serum activity (48–50) and risk of vascular disease (29,30,51–57). We examined several of these variants including −107[A/G] (=SNP9), R192Q (=SNP3) and L55M (=SNP7). The C311S missense coding SNP in PON2 (=SNP19) was previously implicated in cardiovascular disorders, AD and vascular dementia (32,34,38,43). Although none of these SNPs was individually associated with AD, we observed significant association for haplotypes comprising −107[A/G], L55M and C311S. This suggests that −107[A/G], R192Q, L55M and C311S may not be functional determinants of AD risk, but rather in LD with proximate SNP(s) that directly modulate disease pathogenesis. This explanation could account for the inconsistent pattern of association across studies of these SNPs with AD.

Table 2. SNPs genotyped in the PON locus

	NCBI reference	Position (NCBI_35)	Genomic context or predicted function	Gene	Minor allele (frequency) African Americans	Minor allele (frequency) Caucasians	Orientation[a] (observed alleles)
1	rs2237582	94578851	intron	PON1	A (0.29)	G (0.30)	Forward [A/G]
2	rs2269829	94580780	intron	PON1	G (0.49)	G (0.29)	Forward [A/G]
3	rs662	94582097	R192Q	PON1	T (0.32)	C (0.29)	Reverse [C/T]
4	rs2299255	94583437	intron	PON1	C (0.10)	C (0.15)	Forward [C/T]
5	rs13306698	94585433	R160G	PON1	A (0.00)	A (0.00)	Forward [G/[c]]
6	rs1157745	94585689	intron	PON1	G (0.32)	T (0.30)	Forward [G/T]
7	rs854560	94590735	L55M	PON1	T (0.20)	T (0.35)	Forward [A/T]
8	rs854565	94592995	intron	PON1	A (0.35)	A (0.31)	Forward [A/G]
9	rs705379	94598546	promoter (−107)[b]	PON1	A (0.12)	A (0.49)	Forward [A/G]
10	rs705381	94598600	promoter (−161)[b]	PON1	T (0.42)	T (0.26)	Forward [C/T]
11	rs2375001	94609291	intergenic	Intergenic	T (0.22)	T (0.23)	Forward [A/T]
12	rs1859121	94621618	intergenic	Intergenic	T (0.44)	C (0.44)	Forward [C/T]
13	rs2074352	94634324	intron	PON3	T (0.06)	T (0.19)	Forward [C/T]
14	rs3757708	94641564	intron	PON3	T (0.43)	G (0.44)	Reverse [G/T]
15	rs2375003	94646184	N107D	PON3	A (0.00)	A (0.00)	Reverse [G/[c]]
16	rs978903	94648818	intron	PON3	A (0.43)	G (0.43)	Forward [A/G]
17	rs10487132	94664956	intron	PON3	G (0.14)	G (0.45)	Forward [A/G]
18	rs2072200	94670811	promoter	PON3	C (0.07)	C (0.18)	Forward [C/G]
19	rs6954345	94679426	C311S	PON2	C (0.29)	C (0.24)	Forward [C/G]
20	rs3735586	94680233	intron	PON2	T (0.31)	T (0.24)	Reverse [A/T]
21	rs10487133	94680601	intron	PON2	G (0.20)	G (0.10)	Forward [G/T]
22	rs2375005	94681527	intron	PON2	T (0.46)	A (0.44)	Forward [A/T]
23	rs987539	94681643	intron	PON2	C (0.36)	T (0.45)	Forward [C/T]
24	rs6961624	94682312	intron	PON2	G (0.30)	G (0.24)	Reverse [A/G]
25	rs2299263	94685062	intron	PON2	T (0.29)	T (0.25)	Forward [C/T]
26	rs1034809	94696303	intron	PON2	A (0.24)	A (0.25)	Forward [A/G]
27	rs13306699	94698531	F31S	PON2	A (0.00)	A (0.00)	Reverse [G/[c]]
28	rs2286233	94698908	intron	PON2	T (0.24)	T (0.10)	Forward [A/T]
29	rs2299267	94706572	intron	PON2	G (0.13)	G (0.14)	Forward [A/G]

[a]Orientation relative to the top strand of NCBI's build 35.1 (observed alleles as assayed).
[b]Relative to PON1 translation initiation point in build 35.1.
[c]Minor allele not observed in study sample.

In this study, significant evidence of association with AD was observed for −161[C/T] (=SNP10). This PON1 promoter SNP is located within a potential binding site of the transcription factor NF-I (CTF) (51), and we found a pattern of association in which the T-allele has a deleterious effect, both independently and as part of several haplotypes. This is somewhat unexpected because in transient transfection studies done in hepatocytes, the −161[T] allele, as part of haplotypes, confers a 2–3.6-fold higher reporter expression levels than the C allele (51), and in a study of paraoxonase serum levels the T allele is significantly associated with increased activity (52). In the context of vascular disease, it is evident that reduced levels of serum paraoxonase activity are deleterious (24,58–60); however, it remains to be clarified whether AD is associated with elevated or reduced levels of serum paraoxonase (39,41), and which PON1 promoter haplotypes may be responsible for this association.

Overall, we found evidence clearly implicating the PON genes as harboring variants that contribute to AD risk. However, the patterns of association in our study, as well as those in other studies, appear to be complex. This complexity may be due to differences among ethnic groups in the genetic basis of AD. In the Caucasian families, the association signal is strongest with variants in the PON1 promoter, whereas in the African American families, the signal is evident primarily in transcribed regions of PON2 and PON3. However, FBAT analyses of the combined group of families revealed an even stronger signal with SNP10 than in the Caucasian families alone suggesting that the pattern of association with this SNP is the same in African Americans. The lack of a significant association in the African American families when analyzed separately is likely due to the smaller sample size in this group. The complex patterns of association revealed in our study may also be ascribed to a failure to analyze a sufficient number of SNPs within the region necessary to discriminate causative SNPs from association signals arising in neighboring non-causal SNPs. Further genotyping efforts in combination with functional genomic studies of intermediate phenotypes should help clarify the functional role of paraoxonase in AD. Evidence for association in the Caucasian families in only a very narrow portion of the PON cluster (i.e. with haplotypes including SNP10) may account for failure to detect linkage to this region of chromosome 7 in several large genome scans using microsatellite markers (61–64). It is unclear whether the minor linkage peak detected by Pericak-Vance *et al*. (61) with a marker at 7q31.31 is explained by PON.

The involvement of vascular risk factors in AD is well supported by genetic, epidemiological, autopsy and neuroimaging studies (5,6,8–10,18,65–67). Our results and those of other

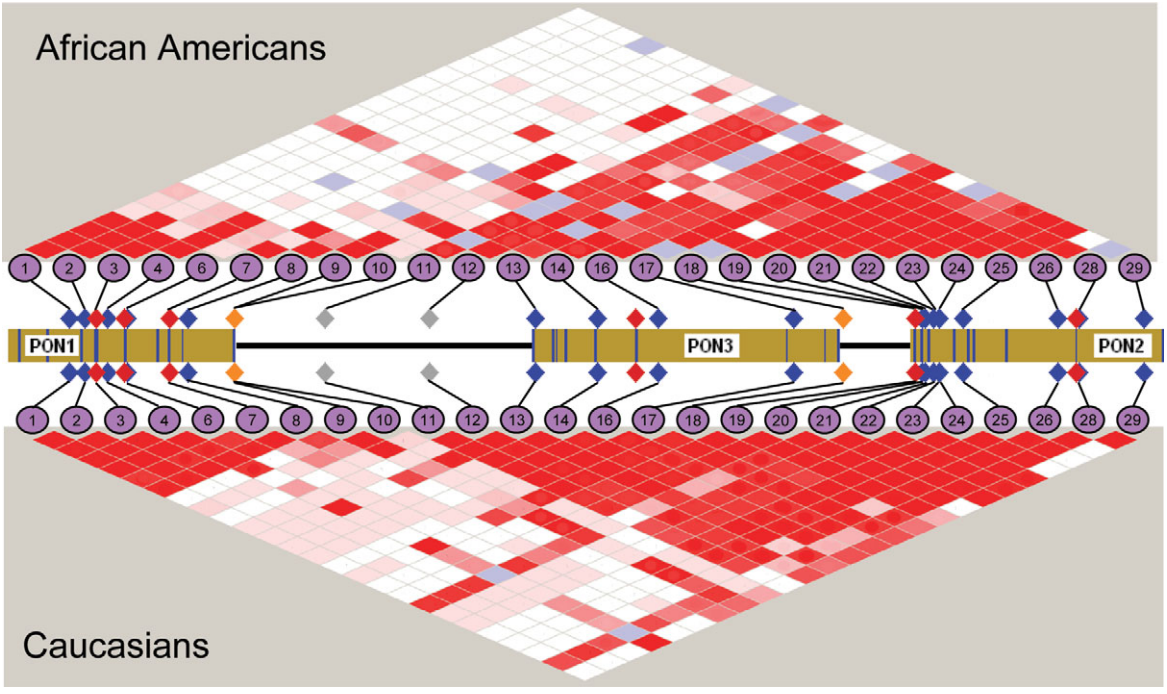

Figure 1. LD in the PON gene cluster. A scheme of the PON locus is shown, with LD maps for African Americans located above and Caucasians below the gene structure. SNPs 5, 15 and 27 were not polymorphic and therefore not included. The predicted functional significance of each SNP is denoted by the symbol color: red, cSNP; blue, intron; orange, 5′ untranslated and gray, intergenic. The measure of LD (D′) among all possible pairs of SNPs is shown graphically according to the shade of red where white represents very low D′ and dark red represents very high D′. High D′ estimates associated with a large confidence interval (most likely due to one of the alleles being rare) are denoted by blue squares.

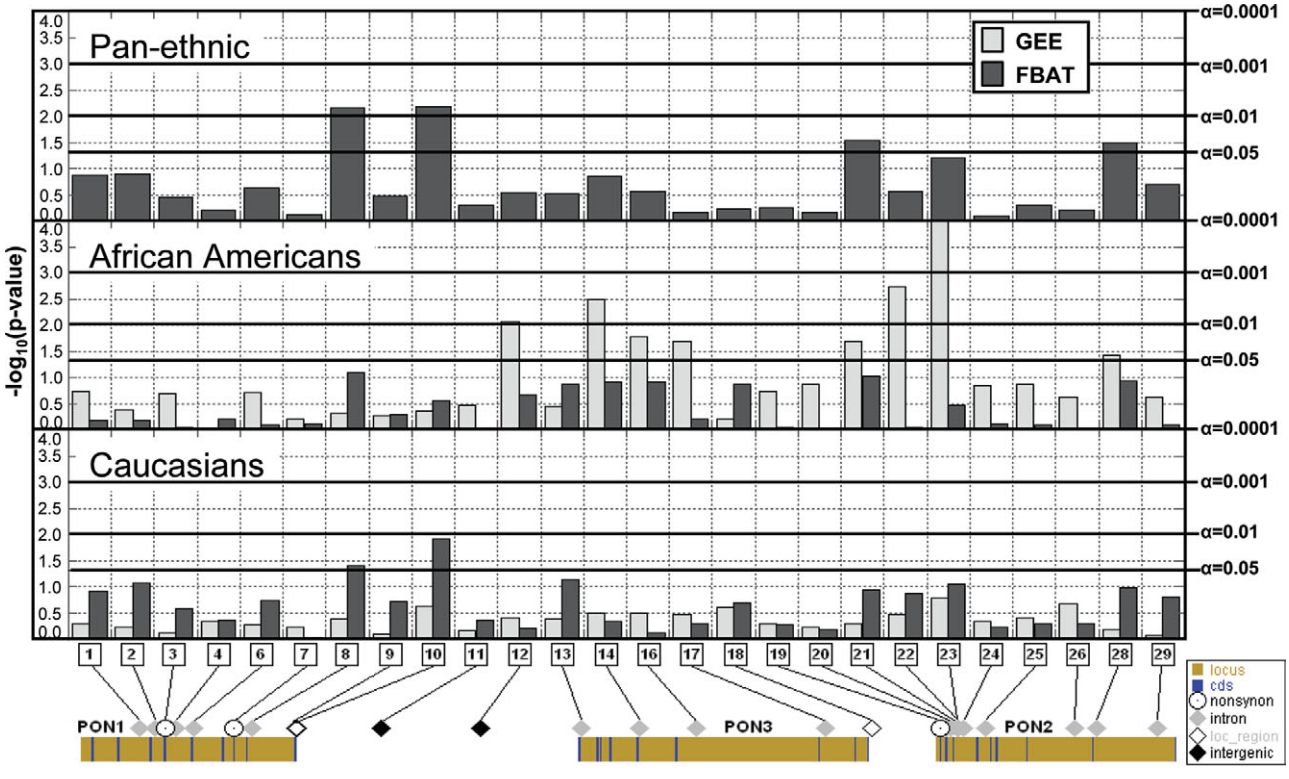

Figure 2. Association of PON SNP genotypes with AD. For each SNP in the PON gene cluster shown at the bottom, negative log *P*-values for tests of association from the GEE (using all data) and FBAT (using sibship data only) analyses are presented for African Americans, Caucasians and pan-ethnic samples. Negative log *P*-values corresponding to significance levels of 0.05, 0.01, 0.001 and 0.0001 are indicated by horizontal lines.

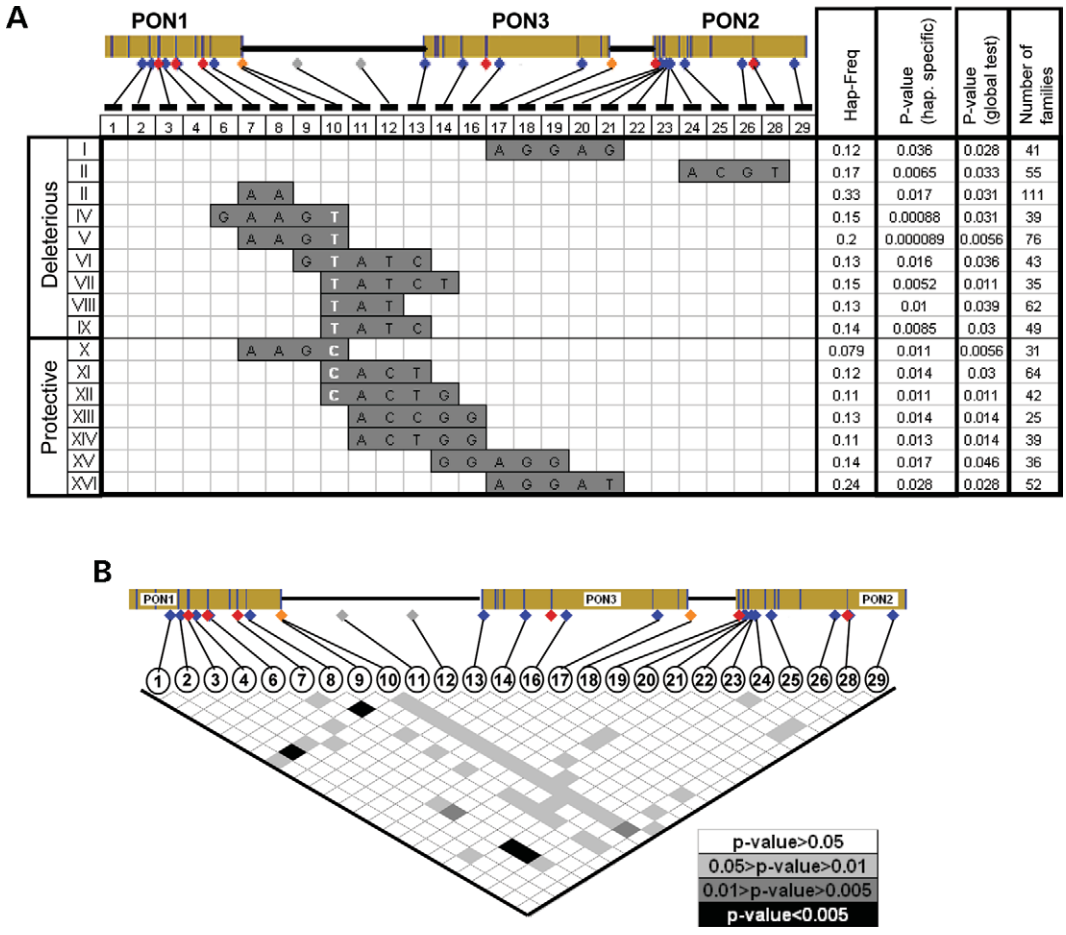

Figure 3. Association of PON haplotypes with AD. The predicted functional significance of each SNP is indicated as described in Figure 1. (**A**) Haplotype analysis of contiguous SNP subsets was performed using the HBAT subroutine of the FBAT software in the pan-ethnic sibships. Haplotypes shown are ones yielding *P*-values ≤ 0.05 in both the global and haplotype-specific tests and are sorted according to whether they are associated with increased or decreased risk of AD. (**B**) All SNP pair combinations were tested for association using HBAT. Global test *P*-values for each SNP pair are represented according to the color code shown beside the grid.

investigators (28–34,38–43) suggest that variations in the PON genes influence risk of vascular disease and AD; however, it is unclear if specific molecular mechanism(s) leading to these different disorders are the same. In the setting of cardiovascular disorders, atherosclerosis of large vessels is clearly consistent with disease etiology. This pattern might not be the case in AD in which the more prevalent vascular pathologies seem to involve small vessels (14). It is also possible that the effect of paraoxonase on AD susceptibility might be exerted through a mechanism that is altogether independent of vascular effects, for example, protection against environmental exposures to neurotoxic organophosphate pesticides (68).

Identification of vascular risk factors for AD is a focus of many current research efforts. Such markers may be used in preclinical diagnosis, and if modifiable can also serve as targets for preventive treatment. In this regard, a recent study suggests that the R192Q polymorphism in PON1 modulates response to cholinesterase inhibitors used in treating AD (69); however, the sample size was small and the threshold for positive response to treatment was low. Nonetheless, this finding highlights the importance of further investigation of the role of paraoxonase in AD.

MATERIALS AND METHODS

Subjects and data collection

The MIRAGE Study is a multi-center family-based study of genetic and environmental risk factors for AD. Details of data collection procedures, protocols for obtaining family histories and reliability of questionnaires have been published elsewhere (70–72). In brief, probands were ascertained at 17 sites in the USA (14), Canada (1), Germany (1) and Greece (1) through research registries or specialized memory clinics. All AD cases were living individuals with probable AD or recently deceased individuals with definite AD verified by brain autopsy. AD was diagnosed in accordance with the NINCDS/ADRDA criteria for probable or definite AD (73). Medical history and risk factor information and blood samples were obtained from AD patients and their available siblings and spouses after obtaining informed consent from

the non-demented subjects and a combination of consent or assent along with informed consent by proxy on living demented subjects. In some instances, when non-demented siblings were unavailable, neighborhood controls of similar ethnic background and living near the AD proband were also enrolled in the study. Cognitive status of individuals identified as non-demented was confirmed by administration of the modified Telephone Interview of Cognitive Status (74). Subjects were classified as African American or Caucasian according to reported ethnicity of the parents. Characteristics of the subjects are shown in Table 1.

SNP selection and genotyping

Twenty-nine SNPs were selected from NCBI's SNP database based on: (1) prior implication in disease, (2) predicted function and genomic context, (3) minor allele frequency, (4) LD structure in the locus, (5) quality of validation evidence and (6) compatibility with the genotyping platform (Table 2). Genomic DNA was extracted from peripheral blood lymphocytes using standard techniques. High throughput genotyping was performed on an ABI 7900 (real-time) platform using the manufacturer's protocols. Errors in genotype data were identified and resolved in several ways: (1) duplicate wells were scattered on DNA template plates and their discordance rate was monitored. Duplicate discordance rate did not exceed 5% and was persistently localized to two samples, which were subsequently excluded, (2) the overall genotype call rate was monitored and was found to be >95% for all 29 SNPs typed, (3) SNP genotypes were tested for compliance with Hardy–Weinberg expectation in a set of unrelated controls in each ethnic group using a χ^2 test. Of the 29 SNPs in the PON gene cluster studied, SNPs 5, 15 and 27 were monomorphic and thus excluded from further analysis. Genotype frequencies for the 26 informative SNPs met Hardy–Weinberg expectation in spouses and unrelated controls, tested separately in each ethnic group.

Statistical methods

Data for individual SNPs were analyzed in two ways. The software package FBAT v1.55 was used to analyze the sibship data (75). These analyses were predicated on the null hypothesis of no linkage and no association. Biallelic tests were performed for SNPs using an additive genetic model. As a second approach, the odds of AD associated with particular marker genotypes taking into account age and gender were computed using a logistic model with the GEE approach (76) implemented in SAS. This approach allows for the correlation structure among relatives and testing of covariates (e.g. age, gender and APOE genotype), and unlike the FBAT analyses, incorporates information from all genotyped subjects in the data set rather than just the families with at least one discordant sib pair with informative genotypes. However, unlike FBAT, GEE does not consider transmission of alleles or identity by descent relationships. Nominal *P*-values are reported for all tests. The Caucasian and African American subgroups are independent samples. Owing to different population histories, unless we genotype functional mutations that are present in both samples, one would not necessarily expect to see the same associations in both samples. However, when polymorphisms within a gene are significantly associated in both samples, this result constitutes a gene-level replication of association.

The association between AD and haplotypes was assessed using the HBAT function of the FBAT software package (77) utilizing a sliding window approach, in which groups comprising 2–5 contiguous markers are tested. Global tests (i.e. multi-allelic mode) were performed first to test the overall association for each group of adjacent SNPs. When the global test of association was significant at the 0.05 level, the corresponding group of SNPs was further investigated using haplotype-specific tests (biallelic mode). Reported *P*-values are asymptotic χ^2 distribution probabilities, which did not differ appreciably from permutation test *P*-values calculated using the $<-P>$ option based on 10 000 Monte Carlo samples from the null distribution. Haplotype global tests for all pairwise combinations of SNPs were also performed.

The LD structure in the PON gene cluster was examined with the program Haploview (http://www.broad.mit.edu/mpg/haploview/documentation.php). Haplotype blocks were defined using confidence-intervals algorithm (78). The default settings were used in these analyses, which create 95% confidence bounds on D′ to define SNP pairs in strong LD. Haplotypes and their frequencies were estimated using an accelerated expectation-maximization algorithm similar to the partition/ligation method (79) implemented in Haploview.

Links to database information

dbSNP database (build 124): http://www.ncbi.nlm.nih.gov/snp.

Annotated genome of chromosome 7 (build 35.1): http://www.ncbi.nlm.nih.gov/mapview.

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Conflict of Interest statement. None declared.

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