Neurogenetics (2009) 10:19–25
DOI 10.1007/s10048-008-0151-3

ORIGINAL ARTICLE

Assessment of Alzheimer's disease case–control associations using family-based methods

Brit-Maren M. Schjeide · Matthew B. McQueen · Kristina Mullin · Jason DiVito · Meghan F. Hogan · Michele Parkinson · Basavaraj Hooli · Christoph Lange · Deborah Blacker · Rudolph E. Tanzi · Lars Bertram

Received: 11 July 2008 / Accepted: 3 September 2008 / Published online: 2 October 2008
© Springer-Verlag 2008

Abstract The genetics of Alzheimer's disease (AD) is heterogeneous and remains only ill-defined. We have recently created a freely available and continuously updated online database (AlzGene; http://www.alzgene.org) for which we collect all published genetic association studies in AD and perform systematic meta-analyses on all polymorphisms with sufficient genotype data. In this study, we tested 27 genes (*ACE*, *BDNF*, *CH25H*, *CHRNB2*, *CST3*, *CTSD*, *DAPK1*, *GALP*, *hCG2039140*, *IL1B*, *LMNA*, *LOC439999*, *LOC651924*, *MAPT*, *MTHFR*, *MYH13*, *PCK1*, *PGBD1*, *PRNP*, *PSEN1*, *SORCS1*, *SORL1*, *TF*, *TFAM*, *TNK1*, *GWA_14q32.13*, and *GWA_7p15.2*), all showing significant association with AD risk in the AlzGene meta-analyses, in a large collection of family-based samples comprised of 4,180 subjects from over 1,300 pedigrees. Overall, we observe significant association with risk for AD and polymorphisms in *ACE*, *CHRNB2*, *TF*, and an as yet uncharacterized locus on chromosome 7p15.2 [rs1859849]. For all four loci, the association was observed with the same alleles as in the AlzGene meta-analyses. The convergence of case–control and family-based findings suggests that these loci currently represent the most promising AD gene candidates. Further fine-mapping and functional analyses are warranted to elucidate the potential biochemical mechanisms and epidemiological relevance of these genes.

Keywords Alzheimer's disease · Risk factors · Genetic association · Meta-analysis · Family-based association testing

Electronic supplementary material The online version of this article (doi:10.1007/s10048-008-0151-3) contains supplementary material, which is available to authorized users.

B.-M. M. Schjeide · K. Mullin · J. DiVito · M. F. Hogan · M. Parkinson · B. Hooli · R. E. Tanzi · L. Bertram (✉)
Genetics and Aging Research Unit, MassGeneral Institute for Neurodegenerative Disease (MIND), Department of Neurology, Massachusetts General Hospital, MGH-East (MIND), 114 16th St., Charlestown, MA 02129, USA
e-mail: bertram.lars@gmail.com

M. B. McQueen · D. Blacker
Department of Epidemiology, Harvard School of Public Health, Boston, MA

M. B. McQueen
Institute for Behavioral Genetics, University of Colorado, Boulder, CO, USA

C. Lange
Department of Biostatistics, Harvard School of Public Health, Boston, MA, USA

D. Blacker
Gerontology Research Unit, Department of Psychiatry, Massachusetts General Hospital, Charlestown, MA, USA

Introduction

Alzheimer's disease (AD) is a genetically complex disorder characterized by neurodegeneration and progressive cognitive dysfunction. Risk for AD is likely influenced by a variety of genes affecting multiple biological pathways. In an effort to identify these susceptibility genes, well over 1,000 genetic association studies have been published implicating or refuting nearly 600 different loci as potential AD loci. Even for the specialist, this wealth of information is becoming increasingly more difficult to follow, much less to interpret. We recently reported the creation of a publicly

available online database, AlzGene, which provides a continuously updated comprehensive summary of published genetic association findings in the domain of AD [1]. One integral part of AlzGene is the calculation of systematic random-effects meta-analyses using published genotype data from eligible case–control studies (i.e., those published in peer-reviewed journals available in English, for more details see [1]).

Using all data available in AlzGene on December 1, 2007, we identified 41 genetic variants in 27 non *APOE*-related genes (see Table 1 for details) showing modest but nominally significant effects on AD risk. While many of these variants have been thoroughly tested across relatively large numbers of independent case–control samples [median=6 (across all meta-analyses), range 4–46; see http://www.alzgene.org for up to date numbers and sample sizes], only seven of the non *APOE*-related loci were also previously assessed in AD family-based samples (e.g. [2, 3]), which may be genetically different from unrelated, population-based cases and controls. However, genuine effects on disease risk should be detectable by both approaches. Family-based methods have the advantage of being robust against bias due to undetected population stratification and phenotype misspecifications [4], which may have affected some of the case–control meta-analysis results. In this study, we tested 29 polymorphisms in the 27 previously implicated genes and two variants in *APOE* (the only currently established genetic risk factor for late-onset AD) for association with AD in four independent collections of AD families with a total of 4,180 individuals.

Materials and methods

Samples All four datasets ("CAG", "NIA", "NIMH", and "NCRAD") tested in this project were originally collected for the study of genetic factors in AD (see Table 2 for a summary of sample characteristics). With the exception of the CAG sample, the majority of pedigrees analyzed in this study were nuclear families ascertained on the basis of the multiple affected ones, generally lacking parental genotypes. In addition to containing at least one affected relative pair, many pedigrees also had DNA available from additional affected or unaffected individuals (mostly siblings). The diagnosis of definite, probable, or possible AD was made according to National Institute of Neurological and Communicative Disorders and Stroke/Alzheimer's Disease and Related Disorders Association criteria for the affected in all four samples. Only families in which no affected individual showed an onset age <50 years were included in this paper. The National Institute of Mental Health (NIMH) families were collected as part of the NIMH Genetics Initiative Study [5]. This sample is comprised of a total of 1,528 subjects from 457 families. Only families in which all sampled affected family members showed an onset age ≥50 years and in which DNA was available from at least two affected family members were included in these analyses, i.e., 1,439 individuals from 436 families. Of these, 1,376 individuals from 410 (94%) families were of Caucasian ancestry. The National Institute on Aging (NIA) and National Cell Repository for Alzheimer's Disease (NCRAD) families were obtained from the NCRAD, and ascertainment and collection details can be found at the NCRAD website (http://www.ncrad.org). For this study, we used families with DNA available from at least two first-degree relatives (concordant or discordant) and in which all sampled individuals affected with AD showed onset ages ≥50 years. For the NIA collection, this was comprised of 1,111 samples from 351 pedigrees (Caucasian, 1,040 samples from 329 pedigrees), and for NCRAD, 1,141 samples from 340 pedigrees (Caucasian, 1,106 samples from 330 pedigrees). The CAG families were recruited from multiple NIA-funded Alzheimer's Disease Research Centers under the auspices of the "Consortium on Alzheimer's Genetics" (see [6] for more details). Probands were included only if they had at least one unaffected living sibling willing to participate in this study. As for the other replication samples, only families in which all sampled affected individuals (generally the proband, although some families had two or more affected subjects) had onset ages ≥50 years were included here, i.e., 489 samples from 217 sibships (Caucasian, 483 samples from 215 sibships).

Genotyping One variant per gene was chosen based on its genetic effect size in the meta-analyses, except for *ACE*, where we elected to genotype three variants to better capture the relatively well-characterized haplotype architecture at this locus [7]. For all variants, genotyping was based on individually optimized single-base extension reactions detected by fluorescent polarization (high efficiency fluorescence polarization), as previously described [6]. Briefly, polymerase chain reaction (PCR) primers were designed to yield products between 200 and 400 bp in length and added to ~10 ng of genomic DNA using individually optimized PCR conditions (see genotyping details in Supplementary Table 2). PCR primers and unincorporated deoxyribonucleotide triphosphates (dNTPs) were degraded by the direct addition of exonuclease I (0.1–0.15 U/rxn) and shrimp alkaline phosphatase (1 U/rxn). The single-base extension step was carried out using Thermosequenase (0.4 U/rxn) and the appropriate mix of R110-ddNTP, TAMRA-ddNTP (3 mM), and all four unlabeled ddNTPs (22 or 25 μM) to the Exo1/SAP-treated PCR product. To assess genotyping quality and assure consistency of the genotyping calls, ~10% of the samples were randomly duplicated and called twice.

Table 1 Comparison of AD association findings in case-control vs. family-based samples

Gene/Polymorphism	Ethnic group	Model	Case–control (AlzGene)[b]		Family-based (NIMH, NIA, NCRAD, CAG)[c]				
			OR (95% CI)	*P* value	MAF	Number of fams	OR	X^2	*P* value
ACE[a]	All	C vs. T	0.83 (0.72–0.94)	0.007	0.45	451	1.02	8.52	0.38
rs1800764	Caucasian	C vs. T	0.79 (0.68–0.92)	0.002	0.44	440	1.01	9.04	0.34
ACE[a]	All	I vs. D	1.08 (1.00–1.17)	0.05	0.46	469	0.96	7.3	0.50
rs1799752	Caucasian	I vs. D	1.05 (0.99–1.13)	0.09	0.46	461	0.96	7.1	0.53
ACE[a]	All	T vs. A	0.82 (0.70–0.96)	0.01	0.37	436	0.95	14.3	0.07
rs4291	Caucasian	T vs. A	0.82 (0.70–0.96)	0.01	0.37	425	0.94	14.6	0.07
BDNF	All	A vs. G	1.03 (0.97–1.09)	0.36	0.21	328	0.92	6.4	0.60
rs6265 (V66M)	Caucasian	A vs. G	1.09 (1.00–1.17)	0.04	0.21	322	0.90	5.8	0.67
CH25H	All	T vs. C	1.41 (1.10–1.80)	0.006	0.12	182	1.01	6.2	0.63
rs13500	Caucasian	T vs. C	1.36 (1.05–1.75)	0.02	0.11	178	1.00	6.0	0.65
CHRNB2	**All**	**T vs. G**	**0.67 (0.50–0.90)**	**0.007**	**0.10**	**170**	**0.79**	**17.4**	**0.03**
rs4845378	**Caucasian**	**T vs. G**	**0.69 (0.51–0.94)**	**0.02**	**0.10**	**165**	**0.79**	**18.0**	**0.02**
CST3	All	A vs. G	1.17 (1.04–1.42)	0.01	0.22	317	1.11	11.2	0.19
rs1064039 (A25T)	Caucasian	A vs. G	1.16 (1.00–1.34)	0.04	0.22	309	1.08	10.1	0.26
CTSD	All	T vs. C	1.13 (1.01–1.26)	0.04	0.10	168	0.90	7.3	0.51
rs17571 (A224V)	Caucasian	T vs. C	1.20 (1.01–1.42)	0.04	0.10	165	0.90	7.1	0.53
DAPK1	All	T vs. C	0.87 (0.79–0.95)	0.002	0.38	426	0.99	7.3	0.50
rs4878104	Caucasian	T vs. C	0.87 (0.79–0.95)	0.002	0.38	418	1.00	6.7	0.57
GALP	All	C vs. G	1.21 (1.10–1.33)	0.0001	0.37	438	0.93	4.3	0.83
rs3745833	Caucasian	C vs. G	1.21 (1.10–1.33)	0.0001	0.37	428	0.92	4.1	0.85
GWA_14q32.13	All	A vs. T	0.84 (0.77–0.93)	0.003	0.43	436	0.91	11.6	0.17
rs11622883	Caucasian	A vs. T	0.84 (0.77–0.93)	0.003	0.44	424	0.92	11.7	0.17
GWA_7p15.2	**All**	**C vs. T**	**1.16 (1.00–1.36)**	**0.06**	**0.25**	**345**	**1.28**	**17.8**	**0.02**
rs1859849	**Caucasian**	**C vs. T**	**1.16 (1.00–1.36)**	**0.06**	**0.25**	**335**	**1.26**	**17.5**	**0.03**
hCG2039140	All	T vs. C	1.23 (1.06–1.44)	0.007	0.16	253	0.96	5.8	0.67
rs1903908	Caucasian	T vs. C	1.23 (1.06–1.44)	0.007	0.16	248	0.97	5.9	0.65
IL1B	All	T vs. C	1.18 (1.03–1.34)	0.02	0.25	338	0.91	7.1	0.53
rs1143634 (+3953)	Caucasian	T vs. C	1.18 (1.04–1.35)	0.01	0.25	331	0.90	6.7	0.57
LMNA	All	C vs. T	1.35 (1.12–1.63)	0.001	0.11	157	1.12	11.1	0.19
rs505058	Caucasian	C vs. T	1.35 (1.12–1.63)	0.001	0.10	147	1.11	9.8	0.28
LOC439999	All	G vs. A	1.15 (1.03–1.29)	0.02	0.50	439	1.06	9.0	0.34
rs498055	Caucasian	G vs. A	1.15 (1.03–1.29)	0.02	0.49	432	1.07	8.2	0.41
LOC651924	All	A vs. G?	0.86 (0.77–0.96)	0.009	0.49	447	1.01	9.6	0.30
rs6907175	Caucasian	A vs. G?	0.86 (0.77–0.96)	0.009	0.50	438	0.99	9.1	0.33
MAPT	All	T vs. C	1.30 (1.01–1.67)	0.04	0.23	331	1.11	11.8	0.16
rs2471738 (intron 9)	Caucasian	T vs. C	1.30 (1.01–1.67)	0.04	0.23	325	1.13	12.6	0.13
MTHFR	All	T vs. C	1.11 (1.01–1.22)	0.03	0.35	412	1.03	12.9	0.11
rs1801133	Caucasian	T vs. C	1.01 (0.91–1.11)	0.82	0.35	405	1.04	13.7	0.09
MYH13	All	C vs. T	1.12 (1.00–1.25)	0.04	0.39	447	1.08	12.1	0.14
rs2074877	Caucasian	C vs. T	1.12 (1.00–1.25)	0.04	0.39	430	1.11	12.9	0.11
PCK1	All	G vs. A	1.29 (1.09–1.52)	0.004	0.14	243	0.98	7.5	0.48
rs8192708	Caucasian	G vs. A	1.29 (1.09–1.52)	0.004	0.15	241	1.03	7.9	0.44
PGBD1	All	A vs. G	1.42 (1.13–1.80)	0.003	0.06	96	0.71	4.0	0.85
rs3800324	Caucasian	A vs. G	1.42 (1.13–1.80)	0.003	0.06	92	0.74	3.9	0.86
PRNP	All	G vs. A	0.89 (0.81–0.97)	0.007	0.34	402	1.02	11.7	0.16
rs1799990 (M129V)	Caucasian	G vs. A	0.88 (0.80–0.97)	0.008	0.34	395	1.02	12.7	0.15
PSEN1	All	G vs. T	0.92 (0.85–1.00)	0.06	0.44	442	0.93	11.7	0.16
rs165932 (intron 8)	Caucasian	G vs. T	0.95 (0.88–1.04)	0.27	0.44	441	0.92	12.7	0.12
SORCS1	All	Min vs. Maj	1.24 (1.04–1.48)	0.02	0.13	185	0.96	5.8	0.67
rs600879	Caucasian	Min vs. Maj	1.24 (1.04–1.48)	0.02	0.12	178	0.95	5.3	0.73
SORL1	All	T vs. A	1.13 (1.01–1.27)	0.03	0.32	407	1.03	9.2	0.33
rs3824968	Caucasian	T vs. A	1.19 (1.05–1.35)	0.007	0.32	399	1.02	8.7	0.37
TF	**All**	**C2 vs. C1**	**1.18 (1.04–1.33)**	**0.01**	**0.19**	**295**	**1.17**	**21.1**	**0.007**
rs1049296 (P570S)	**Caucasian**	**C2 vs. C1**	**1.18 (1.01–1.37)**	**0.04**	**0.19**	**291**	**1.15**	**20.4**	**0.009**

Table 1 (continued)

Gene/Polymorphism	Ethnic group	Model	Case–control (AlzGene)[b]		Family-based (NIMH, NIA, NCRAD, CAG)[c]				
			OR (95% CI)	*P* value	MAF	Number of fams	OR	X^2	*P* value
TFAM	All	G vs. A	0.78 (0.67–0.91)	0.002	0.45	433	1.10	2.3	0.97
rs2306604	Caucasian	G vs. A	0.78 (0.67–0.91)	0.002	0.45	428	1.07	2.4	0.97
TNK1	All	A vs. T	0.84 (0.76–0.93)	0.0006	0.45	448	1.00	9.5	0.30
rs1554948	Caucasian	A vs. T	0.84 (0.76–0.93)	0.0006	0.46	434	0.99	9.6	0.29
APOE	**All**	**ε4 vs. ε3**	**3.68 (3.31–4.11)**	**$<1\times10^{-30}$**	**0.40**	**467**	**4.35**	**>285**	**$<1\times10^{-57}$**
ε2/3/4	**Caucasian**	**ε4 vs. ε3**	**3.81 (3.38–4.29)**	**$<1\times10^{-30}$**	**0.39**	**454**	**4.46**	**>285**	**$<1\times10^{-57}$**

Comparison of the combined association evidence of variants in *APOE* and 27 genes showing nominally significant effects on AD risk in the December 1, 2007, freeze of the AlzGene database ([1]; note that studies on the ε2/3/4 *APOE* polymorphism are not updated but based on data presented in [31]). Genes/variants rendered in bold indicate combined family-based results showing a *P* value <0.05 for association with the same allele as in the case–control meta-analyses

MAF minor allele frequency; *Fams* informative families; *OR* summary odds ratio

[a] Note that while single-locus analyses with variants in *ACE* only show marginally significant effects, haplotype-based analyses yield *P* values <0.05 (see Table 4)

[b] AlzGene results based on December 1, 2007, freeze. For more details, please see Table 2 and Supplementary Table 2 of [1]); for up-to-date meta-analysis results on these variants, please consult the AlzGene website (http://www.alzgene.org)

[c] Test statistics are based on combining one-tailed *P*-values for each variant across all four samples using Fisher's combined probability test, which results in an 8 *df* test (see Supplementary Material for more details and Supplementary Table 1 for results in the individual samples)

Statistical analyses To test for association we used the Family Based Association Testing software package (FBAT; v1.7.3; [8]) under an additive model (the best equivalent to the allelic contrasts used in the AlzGene meta-analyses), assigning equal weights to affected and unaffected individuals (offset=0.5). As for AlzGene, analyses were performed on families of all ethnicities and restricted to families of self-reported "Caucasian" ancestry. To combine statistical evidence across the FBAT analyses from each independent dataset, we used Fisher's combined probability test [9]. Since the hypothesis of this study was to test for association dependent on each allele's direction of genetic effect (i.e. "risk" or "protection"; as observed in the AlzGene meta-analyses), all *P* values used in this calculation are one-tailed. *P* values were inversed ($1-P$) for samples where transmission to the affected was observed with the opposite allele as compared to AlzGene. Odds ratios (ORs) were calculated by fitting a conditional logistic regression model to each data set, where family defines the stratum [10]. Following the meta-analytic approach used in AlzGene, summary ORs across all four samples were calculated using the DerSimonian and Laird random effects model [11]. Note that OR calculations in families are problematic by design (since the estimates are conditioned on family relatedness) and cannot be directly compared to those obtained from case–control analyses. Power calculations were done in PBAT (v3.6; [12]) on the combined sample assuming a disease prevalence of 10%. These calculations (Table 3) show that we have excellent power (>90%) to detect allelic ORs of 1.5 or above across most allele frequencies. Power was ~60–75% for ORs of 1.25 for minor allele frequencies of 0.2 or greater.

Results

Overall, genotyping efficiency for all 31 variants was 98.2%, while the error rate (based on ~10% samples run in duplicate) was 0.35%. One marker deviated signifi-

Table 2 Demographic characteristics of the AD family datasets analyzed

Sample	No. families (subjects)	No. women (%)	No. affected [AAO+SD (range)]	No. unaffected [AAE+SD (range)]
NIMH	436 (1,439)	992 (68.9%)	995 [72.4+7.7 (50–97)]	411 [70.0+10.7 (31–93)]
NIA	351 (1,111)	690 (62.1%)	803 [74.1+7.1 (52–98)]	290 [73.3+9.5 (36–94)]
NCRAD	340 (1,141)	730 (64.0%)	741 [71.3+7.6 (50–98)]	300 [71.0+8.4 (39–93)]
CAG	217 (489)	298 (61%)	222 [69.2+9.0 (50–89)]	267 [72.9+8.8 (49–92)]

The majority of families across all samples are of self-reported "Caucasian" ethnicity (NIMH=94%, NIA=94%, NCRAD=97%, CAG=99%). Numbers missing to total subjects when adding affected and unaffected=phenotype unknown. Note that subjects with unknown phenotype are not included in the association statistics but can be used for reconstructing haplotypes within families

Table 3 Power calculations in combined sample related to the range of allelic summary ORs detected by the meta-analyses in AlzGene

Odds ratio	Minor allele frequency in general population			
	0.05	0.10	0.20	0.50
1.10	0.08	0.11	0.16	0.22
1.15	0.13	0.19	0.30	0.39
1.25	**0.26**	**0.43**	**0.63**	**0.74**
1.50	0.72	0.92	0.99	0.99
1.75	0.96	1	1	1

Power after combining all four family datasets (4,180 samples from 1,344 pedigrees) using an additive disease model. An OR of 1.25 (bold) is approximately equivalent to the average OR found in the 41 significant meta-analyses in December 1, 2007, data freeze when excluding likely *APOE*-related effects (see [1] for details). Assumed disease prevalence 10%, α=0.05

cantly from Hardy–Weinberg equilibrium (HWE) at P= 0.001 in the combined sample (rs505058 in *LMNA*), which was due to a significant HWE deviation in the NIMH dataset. However, the—overall insignificant—results for this single nucleotide polymorphism (SNP) did not change appreciably upon exclusion of this sample from the combined analyses.

Apart from *APOE*-ε4, which was significantly associated in all four datasets (all individual P values$\leq 2\times 10^{-15}$, combined $P<1\times 10^{-57}$), each sample showed nominally significant associations for at least one of the 29 other polymorphisms (Supplementary Table 1), and in many cases, the association was observed with the same allele as in the AlzGene meta-analyses (shaded rows in Supplementary Table 1). However, no single variant besides *APOE* showed nominal evidence of association in more than one sample at a time. This picture is very similar to the results obtained with the individual case–control samples in AlzGene, where non-significant study-specific ORs outweighed significant results in all analyses (see Supplementary Figure 2 of [1]). Upon combining the results of all four family samples, three variants emerged, which showed nominally significant associations with the same allele over- or undertransmitted as in the case–control meta-analyses [rs1049296 in *TF* (P_{ALL}=0.007), rs4845378 in *CHRNB2* ($P_{\mathrm{CAUCASIAN}}$=0.02), and rs1859849 in GWA_7p15.2 ($P_{\mathrm{CAUCASIAN}}$=0.02), Table 1]. Two additional variants showed at least a trend for association in the same direction as AlzGene [rs4291 in *ACE* (P=0.07) and rs1801133 in *MTHFR* ($P_{\mathrm{CAUCASIAN}}$=0.09), Table 1]. Analyzing all three *ACE* SNPs jointly revealed that the "C/T-del-A" haplotype (which defines "clade C") was consistently over-transmitted to affected ones in all samples and also showed nominally significant association in the combined analyses (P ~0.02, Table 4).

Discussion

Our study provides the first systematic family-based assessment of genetic association findings that were derived from a large-scale data synthesis and meta-analysis effort of case–control studies across the whole domain of AD. The observed results are remarkable for a number of reasons. First, when judging the case–control meta-analyses of the original AlzGene report (based on a datafreeze on December 1, 2005) by degree of significance, the five genes with the lowest P values were *ACE*, *CHRNB2*, *GAPDHS*, *PSEN1*, and *TF* (see Table 2 of [1]). With the exception of *GAPDHS*, these are also among the most significant findings observed in the analyses in this study using entirely independent samples and a different study design (note that the association with *PSEN1* is only approaching a statistical trend with a P value of 0.12). Second, the only other variant showing significant association in this study is rs1859849 in GWA_7p15.2, which was originally identified in the first genome-wide association (GWA) analysis published for AD [13]. Although the precise nature and identity of the underlying locus remains elusive, the combination of consistent GWA case–control and follow-up family-based results strongly suggests the presence of a novel AD gene in the vicinity of this marker on the short arm of chromosome 7. Finally, at least two of the non-*APOE* loci associated with AD risk in our family-based samples, i.e., *ACE* and *TF*, were also found to be associated with Aβ levels in cerebrospinal fluid (CSF) in an independent collection of affected and unaffected samples [14]. The independent convergence of (1) significant meta-analysis results in case-control samples, (2) replication of these associations in AD family samples, and (3) a significant genotype-dependent correlation with one of the few established bio-markers in AD strongly implies a genuine disease-risk modifying role of these loci, arguably more so than for any of the other hundreds of suggested AD candidate genes besides *APOE*.

Table 4 Haplotype analyses of variants tested in *ACE*

Sample	Ethnic group	Clade (alleles)	MAF	Fams	X^2	P value[a]
Combined	All	A (T-A-ins)	0.427	489	6.1	0.64
	Caucasian	A (T-A-ins)	0.435	478	6.8	0.56
	All	B (C-T-del)	0.341	457	6.2	0.63
	Caucasian	B (C-T-del)	0.346	449	5.8	0.67
	All	C (C/T-A-del)	0.200	322	17.5	0.025
	Caucasian	C (C/T-A-del)	0.197	315	18.1	0.021

Fisher P value, one-tailed, 8 *df*

While we observed several confirmatory results (in *ACE*, *CHRNB2*, *TF*, and GWA_7p15.2), it should be emphasized that—with the exception of *APOE*—these associations only displayed a modest degree of statistical significance and showed variability of the associations across samples. In the case of *ACE*, statistically significant association was only observed in haplotype-based analyses but not with any of the single markers (best single SNP, P value=0.07, see Table 1). However, given the only modest power to detect ORs below 1.5, lower P values than those observed were beyond the range of this study given the expected genetic effect sizes at the observed allele frequencies. This may also explain that none of the combined results in this study would remain significant after conservative correction for multiple comparisons (e.g., using the Bonferroni method). Note, however, that the goal of this study was to assess whether or not any of the currently most promising AlzGene loci would replicate using family-based methods and not to generate any novel hypotheses that require correction for multiple testing.

ACE encodes angiotensin-converting enzyme-1 (ACE-1), an ubiquitously expressed zinc metalloprotease that is involved in blood pressure regulation. Several epidemiological studies suggest that high mid-life blood pressure may increase the risk for AD in later life [15]. Carriers of the associated clade C have higher plasma levels of ACE-1 as compared to clade A [16]. Furthermore, AD-affected individuals homozygous for clade C have been reported to show elevated CSF Aβ42 levels [17], opposite to what is generally observed in the CSF of AD patients. More recently, ACE-1 activity has been reported to be increased in AD brains proportionately to parenchymal Aβ load [18]. The interpretation of ACE's role in AD pathogenesis is complicated by the observation that it is able to degrade naturally secreted Aβ in vitro [19], which would predict an increased risk in individuals with reduced ACE-1 levels/activity, i.e., opposite to the increased ACE-1 levels expected based on the genetic association observed in this study. It remains to be shown, however, whether ACE's Aβ degrading activity is also relevant in vivo: this was not supported in at least two recent reports [20, 21].

TF encodes transferrin, which is the major circulating glycoprotein involved in iron metabolism and is highly expressed in the brain. There is a vast body of literature suggesting that iron misregulation promotes neurodegeneration, possibly via the generation of reactive oxygen species [22]. In AD, iron has been found to be increased in the brains of AD patients [23], where it is associated with plaques and NFTs [24]. More recently, it was suggested that iron may also play a role in the aggregation of hyperphosphorylated tau into insoluble-paired helical filaments, one of the core ingredients of NFTs [25]. The AD-associated SNP in *TF* constitutes an amino-acid substitution (Pro570Ser), making it tempting to speculate that it may affect the iron-binding properties of transferrin. However, this hypothesis was not confirmed in at least two studies [26, 27], which could either indicate another functional correlate of this SNP or the presence of linkage-disequilibrium with a still elusive AD predisposing variant in or near *TF*.

CHRNB2 encodes the beta-2 nicotinic cholinergic receptor (β2nAChR). Nicotinic acetylcholine receptors are widely expressed in the central nervous system, where the β2nAChR subunit is particularly abundant, forming heteropentameric receptors with α4nAChR subunits (α4β2; [28]). Pathologically, the reduction of nAChRs and the loss of cholinergic neurons in disease-relevant brain regions is one of the major neurochemical hallmarks of AD [29], and several studies have suggested that an age-dependent decrease in protein and/or messenger RNA levels of the α4β2-subtype (in particular β2nAChR) occurs in the cortex and hippocampus of healthy individuals [30]. Although no published studies have directly assessed the functional genomic consequences of the associated variant, it is located only 14 bp 3′ from exon 5 of *CHRNB2*, indicating that it might affect alternative splicing rather than inducing changes in gene/protein expression.

Finally, the GWA_7p15.2 SNP maps close to a predicted gene, NT_007819.514, encoding a protein of 358 residues, whose N terminus exhibits a strong homology to a family of ubiquitin-like proteins, e.g., human ubiquitin C. Thus, the predicted protein possibly plays a role in protein degradation.

In conclusion, the combination of meta-analytically derived case–control association findings with results obtained in this large collection of independent family-based samples suggests that genetic variants in *ACE*, *CHRNB2*, *TF*, and an as yet unknown locus on chromosome 7p15.2 currently appear as the most promising contenders for representing genuine AD susceptibility factors. Further fine-mapping and functional genomic analyses are warranted to elucidate the potential biochemical mechanisms and epidemiological relevance of these genes.

Acknowledgments The authors wish to thank all families for participating in this study. This work was sponsored by grants from the NIA (to L.B.), NIMH (to R.T.), and the Extendicare Foundation (to L.B.). The AlzGene database project is funded by the Cure Alzheimer's Fund (to L.B.).

References

1. Bertram L, McQueen MB, Mullin K, Blacker D, Tanzi RE (2007) Systematic meta-analyses of Alzheimer disease genetic association studies: the AlzGene database. Nat Genet 39:17–23. doi:10.1038/ng1934

2. Bertram L, Blacker D, Crystal A, Mullin K, Keeney D, Jones J et al (2000) Candidate genes showing no evidence for association or linkage with Alzheimer's disease using family-based methodologies. Exp Gerontol 35:1353–1361. doi:10.1016/S0531-5565(00)00193-5
3. Rogaeva E, Meng Y, Lee JH, Gu Y, Kawarai T, Zou F et al (2007) The neuronal sortilin-related receptor SORL1 is genetically associated with Alzheimer disease. Nat Genet 39:168–177. doi:10.1038/ng1943
4. Laird NM, Lange C (2006) Family-based designs in the age of large-scale gene-association studies. Nat Rev Genet 7:385–394. doi:10.1038/nrg1839
5. Blacker D, Haines JL, Rodes L, Terwedow H, Go RC, Harrell LE et al (1997) ApoE-4 and age at onset of Alzheimer's disease: the NIMH genetics initiative. Neurology 48:139–147
6. Bertram L, Hiltunen M, Parkinson M, Ingelsson M, Lange C, Ramasamy K et al (2005) Family-based association between Alzheimer's disease and variants in UBQLN1. N Engl J Med 352:884–894. doi:10.1056/NEJMoa042765
7. Sayed-Tabatabaei FA, Oostra BA, Isaacs A, van Duijn CM, Witteman JC (2006) ACE polymorphisms. Circ Res 98:1123–1133. doi:10.1161/01.RES.0000223145.74217.e7
8. Rabinowitz D, Laird N (2000) A unified approach to adjusting association tests for population admixture with arbitrary pedigree structure and arbitrary missing marker information. Hum Hered 50:211–223. doi:10.1159/000022918
9. Fisher RA (1932) Statistical methods for research workers. Oliver and Boyd, Edinburgh
10. Witte JS, Gauderman WJ, Thomas DC (1999) Asymptotic bias and efficiency in case-control studies of candidate genes and gene-environment interactions: basic family designs. Am J Epidemiol 149:693–705
11. DerSimonian R, Laird N (1986) Meta-analysis in clinical trials. Control Clin Trials 7:177–188. doi:10.1016/0197-2456(86)90046-2
12. Lange C, DeMeo D, Silverman EK, Weiss ST, Laird NM (2004) PBAT: tools for family-based association studies. Am J Hum Genet 74:367–369. doi:10.1086/381563
13. Grupe A, Abraham R, Li Y, Rowland C, Hollingworth P, Morgan A et al (2007) Evidence for novel susceptibility genes for late-onset Alzheimer's disease from a genome-wide association study of putative functional variants. Hum Mol Genet 16:865–873. doi:10.1093/hmg/ddm031
14. Kauwe JS, Wang J, Mayo K, Morris JC, Fagan AM, Holtzman DM, Goate AM (2009) Alzheimer's disease risk variants show association with cerebrospinal fluid amyloid beta. Neurogenetics. doi:10.1007/s10048-008-0150-4
15. Takeda S, Sato N, Ogihara T, Morishita R (2008) The renin-angiotensin system, hypertension and cognitive dysfunction in Alzheimer's disease: new therapeutic potential. Front Biosci 13:2253–2265. doi:10.2741/2839
16. Keavney B, McKenzie CA, Connell JM, Julier C, Ratcliffe PJ, Sobel E et al (1998) Measured haplotype analysis of the angiotensin-I converting enzyme gene. Hum Mol Genet 7:1745–1751. doi:10.1093/hmg/7.11.1745
17. Kehoe PG, Katzov H, Feuk L, Bennet AM, Johansson B, Wiman B et al (2003) Haplotypes extending across ACE are associated with Alzheimer's disease. Hum Mol Genet 12:859–867. doi:10.1093/hmg/ddg094
18. Miners JS, Ashby E, Van Helmond Z, Chalmers KA, Palmer LE, Love S et al (2008) Angiotensin-converting enzyme (ACE) levels and activity in Alzheimer's disease, and relationship of perivascular ACE-1 to cerebral amyloid angiopathy. Neuropathol Appl Neurobiol 34:181–193. doi:10.1111/j.1365–2990.2007.00885.x
19. Hu J, Igarashi A, Kamata M, Nakagawa H (2001) Angiotensin-converting enzyme degrades Alzheimer amyloid beta-peptide (A beta); retards A beta aggregation, deposition, fibril formation; and inhibits cytotoxicity. J Biol Chem 276:47863–47868
20. Eckman EA, Adams SK, Troendle FJ, Stodola BA, Kahn MA, Fauq AH et al (2006) Regulation of steady-state beta-amyloid levels in the brain by neprilysin and endothelin-converting enzyme but not angiotensin-converting enzyme. J Biol Chem 281:30471–30478. doi:10.1074/jbc.M605827200
21. Hemming ML, Selkoe DJ, Farris W (2007) Effects of prolonged angiotensin-converting enzyme inhibitor treatment on amyloid beta-protein metabolism in mouse models of Alzheimer disease. Neurobiol Dis 26:273–281
22. Brewer GJ (2007) Iron and copper toxicity in diseases of aging, particularly atherosclerosis and Alzheimer's disease. Exp Biol Med (Maywood) 232:323–335
23. Loeffler DA, Connor JR, Juneau PL, Snyder BS, Kanaley L, DeMaggio AJ et al (1995) Transferrin and iron in normal, Alzheimer's disease, and Parkinson's disease brain regions. J Neurochem 65:710–724
24. Smith MA, Harris PL, Sayre LM, Perry G (1997) Iron accumulation in Alzheimer disease is a source of redox-generated free radicals. Proc Natl Acad Sci U S A 94:9866–9868. doi:10.1073/pnas.94.18.9866
25. Yamamoto A, Shin RW, Hasegawa K, Naiki H, Sato H, Yoshimasu F et al (2002) Iron (III) induces aggregation of hyperphosphorylated tau and its reduction to iron (II) reverses the aggregation: implications in the formation of neurofibrillary tangles of Alzheimer's disease. J Neurochem 82:1137–1147
26. Lee PL, Ho NJ, Olson R, Beutler E (1999) The effect of transferrin polymorphisms on iron metabolism. Blood Cells Mol Dis 25:374–379. doi:10.1006/bcmd.1999.0267
27. Zatta P, Messori L, Mauri P, van Rensburg SJ, van Zyl J, Gabrielli S et al (2005) The C2 variant of human serum transferrin retains the iron binding properties of the native protein. Biochim Biophys Acta 1741:264–270
28. Kalamida D, Poulas K, Avramopoulou V, Fostieri E, Lagoumintzis G, Lazaridis K et al (2007) Muscle and neuronal nicotinic acetylcholine receptors. Structure, function and pathogenicity. FEBS J 274:3799–3845. doi:10.1111/j.1742–4658.2007.05935.x
29. Oddo S, LaFerla FM (2006) The role of nicotinic acetylcholine receptors in Alzheimer's disease. J Physiol (Paris) 99:172–179. doi:10.1016/j.jphysparis.2005.12.080
30. Tohgi H, Utsugisawa K, Yoshimura M, Nagane Y, Mihara M (1998) Age-related changes in nicotinic acetylcholine receptor subunits alpha4 and beta2 messenger RNA expression in postmortem human frontal cortex and hippocampus. Neurosci Lett 245:139–142. doi:10.1016/S0304-3940(98)00205-5
31. Farrer LA, Cupples LA, Haines JL, Hyman B, Kukull WA, Mayeux R et al (1997) Effects of age, sex, and ethnicity on the association between apolipoprotein E genotype and Alzheimer disease. A meta-analysis. APOE and Alzheimer Disease Meta Analysis Consortium. JAMA 278:1349–1356. doi:10.1001/jama.278.16.1349