

Follow-Up Study of Susceptibility Loci for Alzheimer's Disease and Onset Age Identified by Genome-Wide Association

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Abstract. Replication of genetic association findings in independent studies represents an important validation tool in the search for susceptibility genes for complex diseases such as Alzheimer's disease (AD). In a well-characterized memory-clinic based study comprising 1078 unrelated AD patients and 652 control individuals, we set out to replicate previously reported genome-wide association of four novel risk SNPs with AD and onset age, with first stage p-values ranging from 0.001 to 0.000004. We obtained evidence for association between rs179943, an intronic SNP in *ATXN1* at 6p22.3, and affection status (OR = 0.63 (95% CI = 0.44–0.90; nominal $p = 0.01$)). Overall, our data provided independent support for association of at least one chromosomal locus with AD and warranted a more in-depth investigation of these regions for possible underlying functional variants.

Keywords: Alzheimer dementia, genome-wide association, onset age, replication

INTRODUCTION

It has long been recognized that Alzheimer's disease (AD) is a heritable disorder. Whereas a Mendelian, autosomal dominant pattern of inheritance is only present in a minority of patients, twin studies of non-autosomal

dominant AD suggested a heritability of 60–80% [4]. The unraveling of the genetic etiology of the complex form of AD has been the focus of intense investigation over the past decades. After the promising identification of the *APOE* $\epsilon 4$ allele as a strong genetic risk factor for AD, however, advances have been modest (see *AlzGene*, <http://www.alzgene.org/>). In genomewide association studies (GWA) using high density arrays of single nucleotide polymorphisms (SNPs), the *APOE* locus invariably gave the most significant association with AD (<http://www.genome.gov/gwastudies/>) [1]. Nevertheless, less significant associations were also observed and might pinpoint to genuine molecular mechanisms

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underlying the disease, provided that they are not false positive signals. Therefore, replication in independent studies remains a critical step in determining which association signals are real and should be followed up in more detail.

In a recent family-based GWA, four SNPs (rs11159647, rs179943, rs3826656, and rs2049161 on chromosome 14, 6, 19, and 18 respectively) in addition to *APOE* $\epsilon 4$ reached genome-wide significance following a weighted Bonferroni approach [2] in a multivariate analysis of affection status and onset age (p-values ranging from 0.001 to 0.000004) [3]. Follow up of these association signals in additional collections of family-based samples largely substantiated these findings, but support from two sets of publicly available population-based data [5,6] remained limited. In these two studies, genotype data were available for three of the four SNPs, and statistical analyses revealed only nominally significant associations (based on one-tailed p-values) for two SNPs with AD, each in one study population. Moreover, association with onset age could not be determined [3]. Here, we attempted to replicate the association with these four putative risk loci with both AD and onset age in a well-characterized memory-clinic based study of AD, comprising in total 1730 unrelated AD patients and control individuals.

MATERIALS AND METHODS

Study population

This study was approved by the medical ethical committees of the Hospital Network Antwerp (ZNA), the University of Antwerp, and the University Hospitals of Leuven, Belgium. After informed consent, blood samples of patients and control individuals were collected for genetic studies.

The patient group consisted of 1078 AD patients (mean age of onset = 74.3 ± 8.6 years, % females = 66.4), of which the majority was ascertained at the memory clinic of the ZNA Middelheim, Antwerpen, Belgium (SE, PPDD) in the frame of a large prospective study of neurodegenerative and vascular dementia in Flanders, the Dutch-speaking region of Belgium [7,8]. Another subset of patients ($n = 47$) were derived from a prospective study on the molecular genetics of cognitive impairment which was initiated in October 2006, in collaboration with the memory clinic of the University Hospitals of Leuven (UHL), Belgium (RV). Briefly, patients of Belgian ancestry who visited the memory

clinic of the University Hospitals Leuven, and fulfilled international NINCDS/ADRDA criteria for probable AD [9], were asked to participate.

Upon written informed consent of the patient and a legal representative, a dedicated research nurse collected genealogical information (for determination of Belgian ancestry and family history of disease) as well as blood from patients, their spouse, and/or offspring for DNA extraction and storage of lymphoblasts, serum, and plasma. For each patient, detailed medical information and follow-up was available, including Mini-Mental State Examination (MMSE) [10] and neuroimaging (brain computerized tomography (CT), magnetic resonance imaging (MRI), single photon emission computed tomography, fluorodeoxyglucose positron emission tomography (PET), and/or amyloid imaging). Mutation screening of *A β PP*, *PSEN1*, *PSEN2*, *MAPT*, *GRN*, and *PRNP* in the patient group of the memory clinic of the ZNA Middelheim revealed 8 *PSEN1* mutations, 1 *A β PP* mutation, and 2 *PSEN2* mutations (Brouwers et al, unpublished data). No pathogenic mutations were detected in the patient group of the memory clinic of the University Hospitals of Leuven. Both patient groups shared the same ethnicity.

Most patients met the NINCDS/ADRDA criteria of probable AD ($n = 880$), whereas a small number of patients were diagnosed with possible AD ($n = 80$). Autopsy established a pathological diagnosis of definite AD in 82 patients. Based upon the occurrence of at least one first-degree relative suffering from dementia, the disease was considered familial in 21% of the patients. For 89% of the patients, a reliable estimate of onset age was available, determined by the neurologist on the basis of a caregiver interview.

The control group ($n = 652$, mean age at inclusion = 62.7 ± 15.7 years, % females = 58.4) consisted of healthy individuals, without neurological or psychiatric antecedents or with neurological complaints without memory complaints or organic disease involving the central nervous system. Individuals with a positive family history for dementia or an MMSE ≤ 24 were omitted from the study. Population stratification was excluded as previously described [11].

Genotyping

Genomic DNA was extracted from peripheral blood lymphocytes and stored at a minimum concentration of 50 ng/ μ l. PCR and extension primers for SNPs rs11159647, rs179943, rs3826656, and rs2049161 [3]

were designed using Assay Design 3.1 Software (Sequenom, Inc., Hamburg, Germany).

A total of 20 ng of genomic DNA was PCR amplified. Genotyping was performed by Sequenom MassArray[®] assay (Sequenom, Inc., Hamburg, Germany), followed by Matrix-Assisted Laser Desorption/Ionisation Time-Of-Flight (MALDI-TOF) mass spectrometry. Genotypes were scored both automatically (MassArray Typer version 4.0) as well as by two researchers (blinded to disease outcome). A mean genotyping success rate of 99.6% was obtained for AD patients (range = 99.4–99.9) and 98.5% for control individuals (range = 97.6–99.1). Interplate controls showed 100% concordance for all 4 SNPs.

Statistical analyses

The population of 1078 patients and 652 control individuals allow the study to achieve 95% power to detect a common variant with modest risk (MAF = 0.2 and OR of ~ 1.5) (Genetic Power Calculator) [12]. SNP genotype frequencies did not deviate from Hardy-Weinberg equilibrium in the overall group, as determined using the HWE program version 1.20 [13]. Considering the control group separately, genotypes of rs2049161 deviated from Hardy-Weinberg equilibrium ($\chi = 6.36$; $p = 0.001$). Differences in allele and genotype frequencies between AD patients and control individuals were tested using χ^2 statistics. Odds ratios (OR) (calculated relative to the common genotype) with 95% confidence intervals were corrected for gender, *APOE* $\epsilon 4$ genotype (presence of one or two *APOE* $\epsilon 4$ alleles versus the absence of *APOE* $\epsilon 4$ alleles), and age of onset (age at inclusion for control individuals) using a binary logistic regression model.

The effect of SNP genotypes on onset age in patients was assessed using Cox Proportional Hazard Regression, adjusted for gender and *APOE* $\epsilon 4$ genotype. Patients for whom onset age was undetermined (11%) were not included in the analyses, resulting in a patient group of approximately 960 individuals for the SNPs under study. All statistical analyses were performed using SPSS 14.0 for Windows (SPSS, Inc., Chicago, IL). A Bonferroni corrected p -value of 0.05 (based on the number of SNPs analyzed) was considered significant.

RESULTS

At least one SNP, rs179943 at 6p22.3, was associated with affection status in our sample (Table 1). A

slight decrease in frequency of heterozygous carriers in patients versus control individuals was observed for rs179943; heterozygous carriers had an odds ratio of 0.63 (95% CI = 0.44–0.90, Bonferroni corrected $p = 0.04$) to have AD compared to those homozygous for the major allele (C-allele), after adjustment for age, gender, and *APOE* $\epsilon 4$. A comparable trend could be observed for homozygous carriers of the minor T-allele (OR = 0.51, 95% CI = 0.08–3.28); this difference, however, is not significant as sample sizes for these groups are probably too small to achieve accurate estimates. In line with the genotypic data, the allelic association analysis showed a decreased risk for carriers of the T-allele (OR = 0.64, 95% CI = 0.46–0.89, Bonferroni corrected $p = 0.04$).

Although rs2049161 at 18p11.31 deviated from HWE equilibrium in control individuals, individuals homozygous for the minor allele (C-allele) showed nominally significant association with an odds ratio of 2.83 (95% CI = 1.06–7.54, nominal $p = 0.038$) compared to homozygous carriers of the common allele (A-allele), suggesting that the C-allele is the risk allele, but this did not remain significant after Bonferroni correction. Only a trend was observed for carriers of the C-allele (OR = 1.25, 95% CI = 0.99–1.58, $p = 0.06$). Rs179943 and rs2049161 together explain approximately 0.8% of the variance of the trait ($p = 0.016$).

Using onset age as trait characteristic in the patient group, rs2049161 (at 18p11.31) was the only SNP showing a trend toward association after adjustment for gender and *APOE* $\epsilon 4$ state, with both heterozygous CA carriers and carriers of the C-allele having a hazard ratio of 1.18 (CA genotype; 95% CI = 1.03–1.36, Bonferroni corrected $p = 0.08$; C allele; 95% CI = 1.05–1.33, Bonferroni corrected $p = 0.02$). Homozygous carriers of the risk allele (C-allele) had a decreased onset age of 2.6 years (mean onset age 71.8 ± 8.6 years) compared to homozygous carriers of the wt allele (A-allele; mean onset age 74.5 ± 8.9 years). Marker rs11159647 showed a trend towards association with onset age; homozygous carriers of the minor allele (A-allele) have a reduced hazard (HR = 0.84, 95% CI = 0.70–1.01, nominal $p = 0.056$) compared to homozygous carriers of the major allele (G-allele) (Table 2). This was further confirmed by a reduced hazard for carriers of the A-allele (HR = 0.92, 95% CI = 0.84–1.00, nominal $p = 0.05$), but these results did not remain significant after Bonferroni correction.

Table 1
SNP genotype and allele frequencies: Association with disease

Gene ¹	SNP ²	Controls	AD	OR [95% CI]	P value ³	Controls	AD	OR [95% CI]	P value ³	Risk allele This study	Risk allele Bertram et al.	
NT_026437.1360 14q31.2	rs11159647 Intronic	GG	0.24 (158)	0.28 (299)		G	0.51 (662)	0.52 (1124)				
		GA	0.54 (346)	0.49 (526)	0.80 (0.60–1.06)	0.12	A	0.49 (630)	0.48 (1018)	0.39		A-allele
		AA	0.22 (142)	0.23 (246)	0.87 (0.61–1.23)	0.43						
ATXN1 6p22.3	rs179943 Intronic	CC	0.85 (550)	0.89 (963)		C	0.93 (1191)	0.95 (2037)				
		CT	0.14 (91)	0.10 (111)	0.63 (0.44–0.90)	0.01	T	0.07 (97)	0.05 (117)	0.009	C-allele	T-allele
		TT	0.01 (3)	0.003 (3)	0.51 (0.08–3.28)	0.48						
NT_022209.848 19q13.33	rs3826656 Intronic	AA	0.61 (389)	0.59 (637)		A	0.79 (998)	0.77 (1655)				
		AG	0.35 (220)	0.36 (381)	1.01 (0.78–1.30)	0.98	G	0.21 (274)	0.23 (491)	0.77		G-allele
		GG	0.04 (27)	0.05 (55)	1.15 (0.65–2.04)	0.64						
BC040718 18p11.31	rs2049161 Intronic	AA	0.71 (458)	0.68 (731)		A	0.85 (1094)	0.83 (1774)				
		AC	0.28 (178)	0.29 (312)	1.15 (0.88–1.51)	0.30	C	0.15 (190)	0.17 (2148)	0.06	C-allele	C-allele
		CC	0.01 (6)	0.03 (31)	2.83 (1.06–7.54)	0.04						

¹Gene Symbols and chromosome location are given. ²SNP and SNP function are given. Genotype and allele frequencies are shown with absolute numbers in brackets. Calculations of odds ratios, presented with 95% confidence intervals, were performed using the common genotype as reference. ³Nominal P-values were adjusted for gender, APOE status and onset age. Significant p-values are indicated in bold.

Table 2
SNP genotype and allele frequencies: Association with onset age

Gene ¹	SNP ²	AD	AD	HR [95% CI]	P value ³	AD	AD	HR [95% CI]	P value ³	Risk allele This study	Risk allele Bertram et al.
NT_026437.1360 14q31.2	rs11159647 Intronic	GG	0.28 (273)	G	0.53 (1018)						
		GA	0.49 (472)	A	0.47 (902)	0.92 (0.84–1.00)	0.05				A-allele
		AA	0.22 (215)								
ATXN1 6p22.3	rs179943 Intronic	CC	0.89 (859)	C	0.95 (1818)						
		CT	0.10 (100)	T	0.05 (106)	1.13 (0.96–1.19)	0.22				T-allele
		TT	0.003 (3)								
NT_022209.848 19q13.33	rs3826656 Intronic	AA	0.60 (572)	A	0.77 (1484)						
		AG	0.35 (340)	G	0.23 (438)	1.07 (0.96–1.19)	0.23				G-allele
		GG	0.05 (49)								
BC040718 18p11.31	rs2049161 Intronic	AA	0.69 (658)	A	0.83 (1590)						
		AC	0.29 (274)	C	0.17 (330)	1.18 (1.05–1.33)	0.01				C-allele
		CC	0.03 (28)								

¹Gene Symbols and chromosome location are given. ²SNP and SNP function are given. Genotype and allele frequencies are given with absolute numbers in brackets. Calculations of hazard ratios, presented with 95% confidence intervals, were performed using the common genotype as reference. ³Nominal P-values were adjusted for gender and APOE status. Significant p-values are indicated in bold.

DISCUSSION

Using a large Belgian memory-clinic based sample, we attempted to replicate the association with four SNPs and AD susceptibility and onset age, as reported in a recent GWA analysis [3]. This latter study was the first to employ a large collection of family-based samples instead of population-based samples [3], an approach that has the potential of minimizing the number of false positive as well as false negative results because of its robustness to spurious effects of population substructure and less punitive multiple testing correction because of a weighted Bonferroni approach [2,14].

We identified a significant association for at least one SNP (rs179943) in our population. Evidence of association was strongest for rs179943, located in an intron of *ATXN1* on chromosome 6p22.3, and surviving Bonferroni correction for the number of SNPs investigated in our study. Compared to the original report, however, in which the T-allele was associated with both affection status and onset age as a multivariate phenotype, or with affection status only, we found association with the opposite allele (C-allele). Noteworthy, this C-allele was also overrepresented in AD patients of the publicly available TGEN and GSK datasets [5,6], but in these samples it did not reach statistical significance [3]. The ataxin-1 protein, encoded by the *ATXN1* gene (MIM#601556), is present in various brain regions and non-neuronal tissues. Elongation of the CAG trinucleotide repeat in *ATXN1* results in spinocerebellar ataxia (SCA1), in which degeneration of the cerebellum, brainstem, and spinal cord causes the clinical features [15].

A second SNP, rs2049161, an intronic SNP in a gene of unknown function at 18p11.31 (BC040718), was associated with both disease status and onset age in our sample. However, this should be interpreted with caution because this SNP deviated from Hardy-Weinberg equilibrium in the control group, most likely because of low numbers of homozygous carriers of the rare allele (frequency of CC-genotype = 0.9%). Moreover, it should be noted that this association would not survive Bonferroni correction for multiple testing. This SNP was only marginally associated with affection status in the original study sample and in the case-control samples of the GSK dataset showing nominal significance with the same risk allele [3].

Apart from a trend towards association between homozygous carriers for the opposite allele (A-allele of rs11159647) and onset age, we could not replicate the strongest finding of the original GWA study. Nor did

we obtain an association for rs3826656 on chromosome 19. Several reasons can account for these discrepancies between our association findings and those reported in the original study, including chances of false positive or negative findings. Lack of power in our sample is unlikely since our sample size was twice as large as required to detect association with rs11159647 [3], and our power calculations showed sufficient power to detect modest effect sizes.

Due to the fact that our patient and control groups were not perfectly gender- or age-matched, we cannot exclude the possibility that some control individuals might still develop AD later in life. Nonetheless, considering that this will increase the chance of false-negative findings rather than false-positive findings, the association signal we found with rs179943 may have been underestimated. In addition, the logistic regression analyses were adjusted for gender and age.

To further test whether our results might have been biased by the younger control group, we calculated genetic association after removing from the analysis all control individuals aged 50 years or less ($n = 169$). The remaining control group comprised 483 individuals with a mean age at inclusion of 70.4 ± 10.0 years. The association with rs179943 remained significant, with a comparable OR as we had calculated in the total control population (CT genotype; OR = 0.64, 95% CI = 0.45–0.92, nominal $p = 0.015$), lending extra credibility towards our findings. A similar finding was obtained for rs2049161 (CC genotype; OR = 2.80, 95% CI = 1.09–8.12, nominal $p = 0.033$).

No other GWA studies on AD reported a direct association for one of the 4 SNPs under study [16–20]. Differences in study design may explain part of this lack of replication. For example, all other GWAs used population-based rather than family-based samples, and none used a multivariate analysis of AD risk and onset age. One study followed a DNA-pooling strategy which may have affected accuracy of genotype frequency estimates though errors in predicted frequencies were reported to be low [18]. One other study was limited to putative functional SNPs [16] and would therefore have missed all SNPs that were studied here. However, as most replication studies mainly focus on those SNPs that are genome-wide significant or surpass certain criteria for follow-up (e.g., a predefined number of most significant ('top') SNPs), the possibility exists that these 4 SNPs are still associated at a lower significance levels or that other SNPs in LD with those regions are associated. For example, two GWA studies, including one large international collaborative

study, found numerous hits in a 2 Mb region surrounding rs11159647 on chromosome 14 [17,21]. These SNPs are all positioned in predicted genes for which the biological interpretation remains unclear. Of interest, Beecham and colleagues [19] reported an association with rs1402627 (p-value 4.42E-05), positioned at 6 kb from rs2049161 in the same cDNA BC040718 at 18p11.31 [3], and this was the same SNP for which we found tentative evidence of association. Furthermore, Harold et al. [21] found an association with rs16879127 in *APOE* ϵ 4 positive individuals positioned at 2 kb 5' of the *ATXN1* gene, further underscoring a possible role for this gene in AD. Of note is that, although our patient/control sample was included in the latter GWA study, they were only used at stage 2 for replicating the genome-wide significant hits obtained at CLU and PICALM and not for the genome-wide genotyping.

It remains unclear at this stage whether the associations with opposite alleles at the same marker locus should be considered as evidence against a genuine association, or whether they are merely the result from minor population differences in LD structure, allele frequencies, and gene or environment interactions, particularly in light of the small effect sizes observed. However, given that our study population was of European ancestry, like the vast majority of samples used in the original report, this seems less plausible. But, it should be noted that, even in populations of European ancestry, allele frequencies can differ substantially, as seen, e.g., for rs11159647 when comparing genotype frequencies in our Belgian population with those of Utah residents with Northern and Western European ancestry from the CEPH collection of the International HapMap Project (<http://www.hapmap.org/>). Interpreting these effects in meta-analyses will be difficult and requires further investigation of the locus. A detailed comparison with the data of other GWA studies will permit to delineate chromosomal regions that should be targeted for fine-mapping efforts. Lastly, perhaps the original family-based study was enriched for less frequent alleles that we were unable to detect in our study population. Notwithstanding these difficulties, which are inherent to studies of complex diseases and traits, we found independent evidence of association for at least one SNP (rs179943 at chr6p22.3) and a tentative evidence for rs2049161 (at 18p11.31). Although technically, association with the opposite allele in our sample cannot be considered a direct replication of the original finding, the fact that we did find evidence of association at rs179943 suggests that a more in-depth exploration of this chromosomal region is warranted.

If our association signals point to genuine risk factors, they are unlikely to explain a large proportion of the variance, but nevertheless they might uncover important pathomechanisms involved in AD and onset of disease.

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