Olfactory copy number association with age at onset of Alzheimer disease

C.A. Shaw, PhD
Y. Li, MD, PhD
J. Wiszniewska, MD
S. Chasse, PhD
S.N.Y. Zaidi, MD
W. Jin, MD
B. Dawson, MS
K. Wilhelmsen, PhD
J.R. Lupski, MD, PhD
J.W. Belmont, MD, PhD
R.S. Doody, MD, PhD
K. Szigeti, MD, PhD

Address correspondence and reprint requests to Dr. Kinga Szigeti, Department of Neurology, University of Buffalo, SUNY, Buffalo, NY 14203
szigeti@buffalo.edu

ABSTRACT

Objectives: Copy number variants (CNVs) have been recognized as a source of genetic variation that contributes to disease phenotypes. Alzheimer disease (AD) has high heritability for occurrence and age at onset (AAO). We performed a cases-only genome-wide CNV association study for age at onset of AD.

Methods: The discovery case series (n = 40 subjects with AD) was evaluated using array comparative genome hybridization (aCGH). A replication case series (n = 507 subjects with AD) was evaluated using Affymetrix array (n = 243) and multiplex ligation-dependent probe amplification (n = 264). Hazard models related onset age to CNV.

Results: The discovery sample identified a chromosomal segment on 14q11.2 (19.3–19.5 Mb, NCBI build 36, UCSC hg18 March 2006) as a region of interest (genome-wide adjusted p = 0.032) for association with AAO of AD. This region encompasses a cluster of olfactory receptors. The replication sample confirmed the association (p = 0.035). The association was found for each *APOE4* gene dosage (0, 1, and 2).

Conclusion: High copy number in the olfactory receptor region on 14q11.2 is associated with younger age at onset of AD. ***Neurology*® 2011;76:1302–1309**

GLOSSARY

AAO = age at onset; **aCGH** = array comparative genome hybridization; **AD** = Alzheimer disease; **CI** = confidence interval; **CN** = copy number; **CNV** = copy number variant; **DZ** = dizygotic; **FISH** = fluorescent in situ hybridization; **HR** = hazard ratio; **LD** = linkage disequilibrium; **MLPA** = multiplex ligation-dependent probe amplification; **MZ** = monozygotic; **OR** = odds ratio; **RR** = relative risk; **SNP** = single nucleotide polymorphism.

Alzheimer disease (AD) is the most common form of dementia and leads to progressive cognitive decline.[1] The incidence of AD rises from 2.8 per 1,000 person-years in the 65- to 69-year age group to 56.1 per 1,000 person-years in the older than 90 years age group.[1] Heritability for AD has been estimated from genetic epidemiologic studies. Twin studies have shown higher concordance for monozygotic (MZ) than for dizygotic (DZ) twins: the pairwise concordance for AD was 18.6% in MZ pairs and 4.7% in DZ pairs and the corresponding proband-wise concordance rates were 31.3% and 9.3%.[2]

Age at onset (AAO) of AD is an important attribute that merits therapeutic targeting. If the age at disease onset can be delayed by 5 years, it is estimated that the overall public health burden of AD will decrease by one-half by 2047.[3] *APOE* has been found to be an important influence on AAO, and additional loci likely influence AAO of apparently sporadic AD. Genome-wide case-control and AAO association studies using single nucleotide polymorphism (SNP) arrays have identified candidate regions[4] (www.alzgene.org); however, copy number variant (CNV) association studies have not yet been reported in the literature.

The observation of widespread and abundant variation in the copy number (CN) of submicroscopic DNA segments has greatly expanded our understanding of human genetic variation.[5]

Supplemental data at www.neurology.org

From the Departments of Molecular and Human Genetics (C.A.S., Y.L., J.W., W.J., B.D., J.R.L., J.W.B.) and Neurology (Y.L., S.N.Y.Z., R.S.D., K.S.), Baylor College of Medicine, Houston, TX; Departments of Genetics (S.C.) and Neurology (K.W.), The University of North Carolina at Chapel Hill, Chapel Hill; Howard Hughes Medical Institute (B.D.), Houston, TX; and Department of Neurology (K.S.), University of Buffalo, SUNY, Buffalo, NY.

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CNVs confer a novel genetic marker map assaying association signals that are not detectable by SNPs.[6] With the advent of microarray technology allowing genome-wide ascertainment of CNVs, disease associations have been reported in schizophrenia, systemic lupus erythematosus, and HIV susceptibility.[7-9] CNVs influence gene expression, phenotypic variation, and adaptation by altering gene dosage and genome organization.[5,9]

In this study, we performed a genome-wide CNV association study to identify loci that modify AAO of AD.

METHODS

Subject cohorts. The discovery (n = 40) and replication (n = 507) cohorts met National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer's Disease and Related Disorders Association criteria for probable AD.[10] The discovery cohort was ascertained at the AD and Memory Disorders Center of Baylor College of Medicine.[11] Cases found to carry the *APOE*4/4 genotype were excluded from the discovery cohort to diminish the variance in AAO caused by the *APOE*4 allele. The replication dataset was ascertained as part of the Texas Alzheimer Research Consortium project.[12] Further description of the cohorts is available in appendix e-1 and table e-1 on the *Neurology*® Web site at www.neurology.org.

Standard protocol approvals, patient consents. The Institutional Review Board of all participating sites approved the study. Informed consent was obtained from each subject and their legally authorized representative, as subjects with dementia constitute a vulnerable population.

AAO phenotyping. AAO was determined with 2 standardized methods in both cohorts: 1) caregiver estimate by prompted standard question regarding onset of symptoms and 2) physician estimate of duration of illness using a standardized and validated structured interview with landmark event to facilitate recall.[13]

Detection of CN variation association with AAO. ***Discovery cohort.*** The discovery study was exploratory, as there were no available data on CNV and genome-wide association study in AD. The post hoc power analysis for a Weibull regression using our empirical results from this study to determine a distribution of ages and CNV states showed a power of between 40% and 60% to detect an association of this effect size for a risk allele frequency of between 5% and 10% in a sample size of 40.

Array comparative genome hybridization (aCGH) was used to detect genomic CN variation at a high resolution level with an 8.9-kb overall median probe spacing (Agilent Human Genome CGH microarray 244A). The normalized log-ratio data were generated by the manufacturer's microarray scanner and quantification software (CGH analytics, Agilent) and were sorted by genomic location using the NCBI build 36 (UCSC hg18 March 2006) of the human genome. The sorted data were grouped into 5-probe sliding window bins, and the mean log2 ratios for each bin were determined for use in the subsequent analysis.

AAO was related to log2 ratio using Weibull parametric hazard function regression (survival package in R; http://cran.r-project.org). The 5-probe sliding window size was selected empirically based on extensive experience in clinical CNV detection which utilizes confirmation by fluorescent in situ hybridization (FISH). We performed a genome-wide correction for multiple testing by repeating the analysis 1,000 times permuting patient AAO. For each permutation the maximum geometric mean of $-\log p$ values for 20 consecutive bins was determined. The use of 20 consecutive bins incorporates the dependence between the consecutive 5-probe sliding windows as well as our desire to focus on regions that exhibit a consistent association across a genomic region. Only 32 of 1,000 random permutations generated a set of contiguous results where the geometric mean of p values was as strong as that found for the 14q11.2 interval.

In order to rule out spurious association from a rare CNV present in only one individual at the extremes of the AAO spectrum we calculated signal variance for each bin. We determined the 99th percentile of these variances and we identified all regions that exceeded this variance threshold (n ~ 2,400). We applied the 99th percentile variance filter as a second dimension complementary to the p value for hazard regression analysis to prioritize highly variable AAO-associated regions for replication analysis. Furthermore, we performed segmentation analysis on the cohort using the Agilent ADM2 algorithm to confirm that the associations observed in the hazard analysis correspond to actual segmental events. We required that the CNV is present in at least 5% of the cohort (2 or more individuals).

Replication cohort. A replication cohort (n = 507 subjects with AD) was evaluated using Affymetrix array (n = 243) and multiplex ligation-dependent probe amplification (MLPA)[14] (n = 264) (table e-2). The replication cohort sample size was determined based on the effect size from the discovery cohort taking into account the variance of AAO due to the *APOE*4, as the replication cohort included all *APOE*4 genotypes. Power analysis for the replication study was performed using the time-to-event statistical power calculation method *spower* from the survival analysis utility R library Hmisc (http://cran.r-project.org/). We used the observed AAO data from the interval defined in the preliminary data to simulate AAO values for such CNV data (1,000 replicates). For a sample size of 350, we achieve a power of 90% in our simulations.

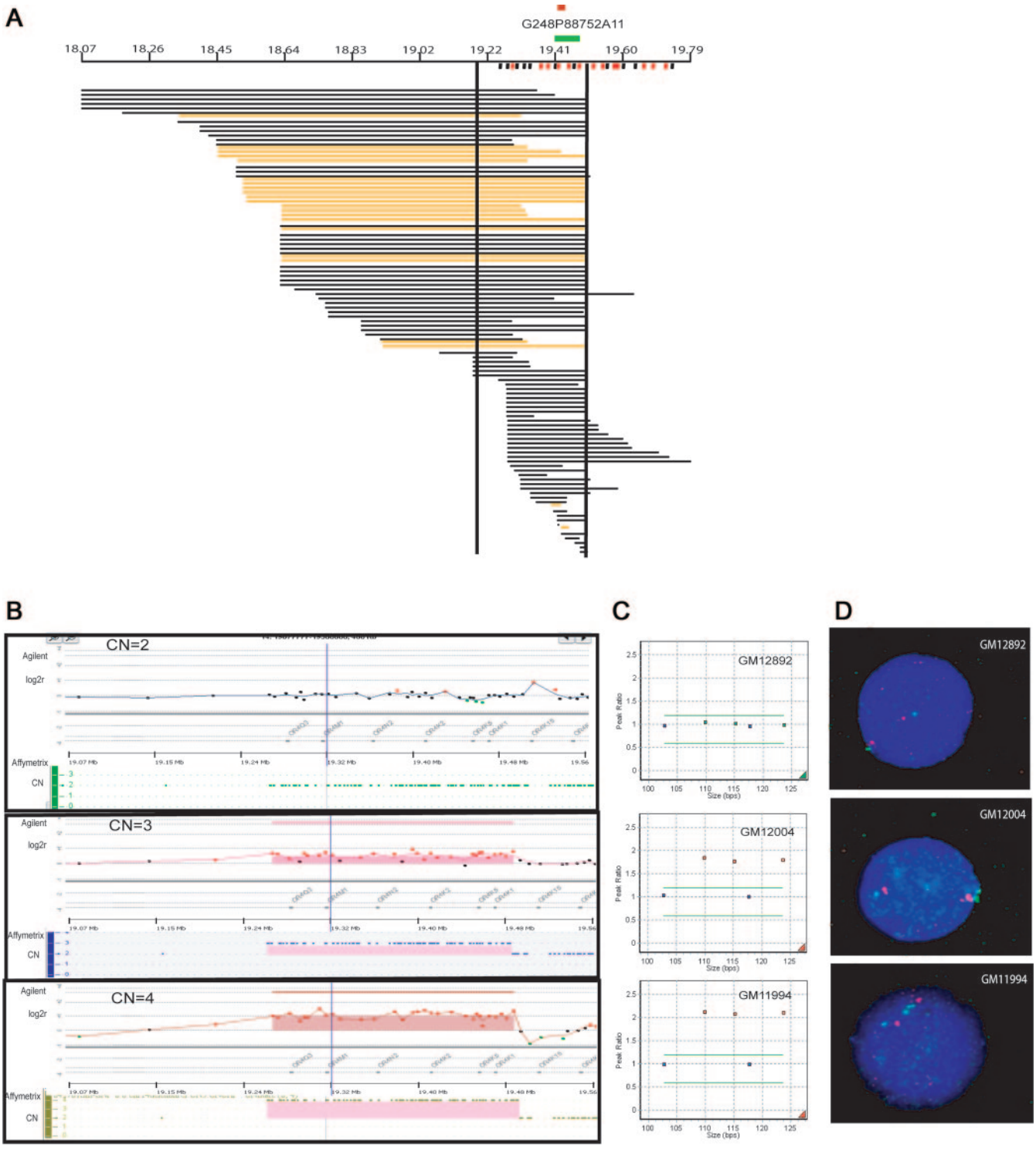
CN genotype for 243 patients was assayed on a Genome-Wide Human SNP Array 6.0 (Affymetrix). The log2ratios were generated in the Genotyping Console 3.0.1 software after regional GC correction using 78 concomitantly ascertained normal controls as reference. A Gaussian mixture model was applied to the normalized mean intensities to analyze CN state (appendix e-1 and figure e-1). The CN calls were validated by MLPA (MRC Holland) in a subset of subjects and in all subjects with the 5+ CN state (figure e-2). CN genotype for the subsequent 264 subjects was assayed by the high throughput MLPA assay. CN calls were also made by use of a Gaussian mixture model (appendix e-1 and figure e-1). The distribution of CN states within the assays is depicted in table e-3.

AAO was related to the inferred CNV dosage of the 14q11.2 region detected in the discovery cohort (Variation_0316; http://projects.tcag.ca/variation/) using Cox proportional hazard regression (Survival Package in R; http://cran.r-project.org/). We incorporated sex, *APOE*4 dosage (0, 1, 2), and the interaction between *APOE*4 dosage and the chromosome 14q11.2 locus CN state as covariates into the regression model.

RESULTS

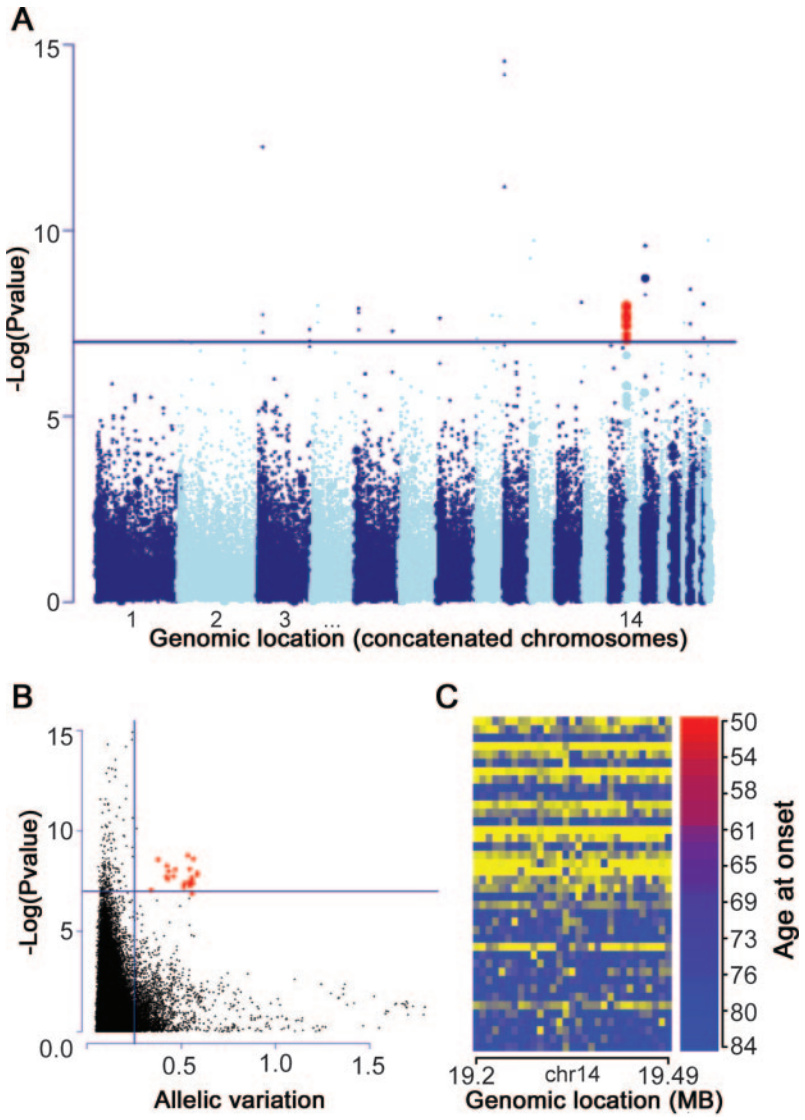
QC and genotyping consistency between platforms and FISH confirmation. QC measures are detailed in appendix e-1. The CNVs inferred in the replication cohort are depicted in figure 1A in the context of previously observed variants in the Data-

Figure 1 The genomic context of variation_0316 and genotyping accuracy



(A) The multiallelic variation_0316 (black bars) detected in 35% of the subjects in relation to known copy number variants (CNVs) (orange bars). The genomic coordinates on chromosome 14 are expressed in Mb. The hashed red bars on the genomic coordinate line are olfactory receptor genes and the black bars represent pseudogenes. (B) Three subjects ascertained on both the array comparative genome hybridization (aCGH) and single nucleotide polymorphism (SNP) arrays, having 2, 3, and 4 copy genotypes in the 3 respective panels. The aCGH data are plotted by $\log_2$ ratio and the SNP array data are visualized as copy number state after segmentation with the HMM algorithm. Multiplex ligation-dependent probe amplification assays are shown in (C) for 3 HapMap samples harboring 2, 3, and 4 copy genotypes (equivalent of panel B). Confirmation of gene dosage and genomic location by fluorescent in situ hybridization using G248P88752A11 fosmid clone (green signal) and RP11-52401 control BAC clone (red) for the same 3 HapMap samples harboring 2, 3, and 4 copy genotypes are demonstrated in (D).

Figure 2 Variation_0316 association with age at onset (AAO) of Alzheimer disease in the discovery cohort



The discovery study is summarized in figure 2. The $-\log_e p$ values for Z scores from the hazard function regression performed on the array data using a 5-probe sliding window are plotted as a function of genomic location (A). In panel B, the $-\log_e p$ values are plotted against the variance (surrogate for allele frequency and number of alleles). The variance filter serves to exclude spurious association from a rare copy number variant not highly observed in the cohort and present in only one or a few individuals at the extremes of the AAO spectrum (B). (C) Age information and array data for AAO is represented in the red and blue color bar to the side of each array data heat map. Each cell in the subject age bar is adjacent to the row of the array data heat map for that subject. Red represents younger subjects, while blue represents older subjects in a linear scale from age 55 to age 84. The blue and yellow heat maps convey copy number array data for the region. Each blue-yellow row represents a subject and each column represents the data for a single oligonucleotide probe. Genomically adjacent oligos are shown next to each other from left to right.

base of Genomic Variants. The sizes and location of the CNVs detected in the replication study are in agreement with the reported CNVs. We validated that CN was congruent between the various assays (Agilent Human Genome CGH microarray 244A, Genome-Wide Human SNP Array 6.0 [Affymetrix], and the MLPA) by genotyping a subset of samples by 2 or 3 assays (figure 1, B and C). The unique sequence around the *OR4K2* gene allowed us to confirm the CNV by FISH[15] (figure 1D). The FISH suggests that up to 3 copies are located on the same chromosome in close proximity.

Olfactory receptor cluster CN association with AAO of AD. ***Discovery cohort.*** AAO was associated with CN variation on chromosome 14q11.2 between 19.2 and 19.49 MB (NCBI build 36, UCSC hg18 March 2006): 22 consecutive results had a $-\log_e p$ value of 7 or more (figure 2A and table e-4) (multiple testing corrected $p = 0.032$). This region also had high log2 ratio variability (top 1%) and segmentation confirmed CNV calls at an allele frequency over 5% (figure 2B). The array data heat map for the region (figure 2C) visually illustrates that higher CN (yellow) is associated with earlier AAO (figure 2C). A 10-year difference in median AAO—67 years vs 77 years—was found when comparing high to low CN partitioned cohort (normalized mean log2 ratio >0 vs <0).

The interval includes a multiallelic cluster of overlapping CNVs with various breakpoints (figure 1A and figure e-2). The association signal comes from olfactory receptor gene cluster in the region where *OR4M1*, *OR4N2*, *OR4K2*, *OR4K5*, and *OR4K1* are located. The summary test statistics (Weibull model) for the region are summarized in table 1. The complete set of hazard function outcomes with $-\log_e p > 7$ depicted in the Manhattan plot are summarized in table e-4. The chromosome 14q11.2 region is the only region that survived multiple testing correction and fulfilled our a priori significance filter settings (allele frequency >5% and variance in >99th percentile).

Replication cohort. The Cox proportional hazard analysis confirmed the association of CNV variation_0316 dosage on chromosome 14q11.2 with AAO of AD ($p = 0.03$) in the replication cohort (tables 1 and 2). The analysis also replicated significant AAO association of *APOE* 4/4 reported in the literature. No interaction between *APOE* and the CN locus was identified by the regression; no gender effects were detected. The dosage association of the olfactory receptor gene cluster on AAO of disease is presented in the box plot (figure 3A). The time to event survivorship curves for the various CN states depicts the percentage of subjects diagnosed by age for each CN state (figure 3B). The relative risk (RR) for onset of AD is proportionally increased 1.12-fold for each one-copy increase of the olfactory receptor CN state (table 1). The association of the 14q locus appears to be independent of and in addition to the effect of *APOE*.

The most striking association signal appears to originate from the 5+ CN state on all *APOE* back-

Table 1 Variation_0316 dosage association with AAO of AD: Weibull hazard outcome in the discovery set and Cox proportional hazard outcomes in the replication cohort

Cohort	Model	Variable	p Value	Relative risk	95% CI
Discovery	Dosage	CN	0.0005153	2.117	1.386-3.232
Replication	Dosage	CN	0.0346015	1.124	1.008-1.252
Replication	Dosage	E4/4	1.25E-09	2.407	1.813-3.195
Replication	Categorical[a]	CN	0.0045201	1.938	1.228-3.061
Meta-analysis	Dosage	CN	0.0092225	1.149	1.035-1.276
Meta-analysis	Dosage	E4/4	2.55E-09	2.322	1.76-3.063
Meta-analysis	Categorical[a]	CN	0.0012671	2.053	1.326-3.179

Abbreviations: AAO = age at onset; AD = Alzheimer disease; CI = confidence interval; CN = copy number.
[a] Categorical model: the data suggest that the 5+ CN state represents the highest risk. We applied a categorical model (category 1 includes 2, 3, and 4 CN states, category 2 the 5+ CN state) to estimate the risk for the 5+ state.

grounds; the 2 copy and 3 copy states have similar AAO and the 4 copy state has a trend toward earlier AAO on all *APOE*4 backgrounds. We built an additional Cox proportional hazard model (categorical) treating the 2, 3, and 4 copy states as a single class and the 5+ state as the other CN class based on this observation. The 5+ CN subjects were 2 times more likely to be diagnosed with AD by any given age compared to the 2, 3, and 4 copy carriers (RR = 2.05, confidence interval [CI] 1.33–3.18, $p = 0.001$) (table 1). The risk of onset before age 72 years (median in the entire sample) for persons who had CN of 5 or more was 6-fold higher compared to persons having lower CN (hazard ratio [HR] = 5.8, 95% CI 1.7–20). The risk for *APOE*4/4 subjects was 4-fold higher compared to other *APOE* genotypes (HR = 4.1, 95% CI 2.3–7.6).

Table 2 Summary of AAO distribution in the CNV variation_0316 dosage groups

Genotype			AAO			
CN	*APOE*4	Subjects (n=507)	Median	Mean	SE	Range
2	N/N	80	73.5	73.19	1.17	48-98
	N/4	110	73	71.95	0.74	52-89
	4/4	29	65	66.55	1.4	48-80
3	N/N	78	76	73.04	1.07	47-86
	N/4	80	74	72.11	0.95	51-89
	4/4	26	69	69.21	1.4	50-86
4	N/N	28	69	69.33	2.06	52-90
	N/4	47	72	71.33	1.22	49-90
	4/4	9	66	65.73	1.53	58-73
5+	N/N	2	65.5	65.5	0.5	65-66
	N/4	13	67	66.35	2.43	53-83
	4/4	5	58	62	3.91	55-76

Abbreviations: AAO = age at onset; CN = copy number; CNV = copy number variant.

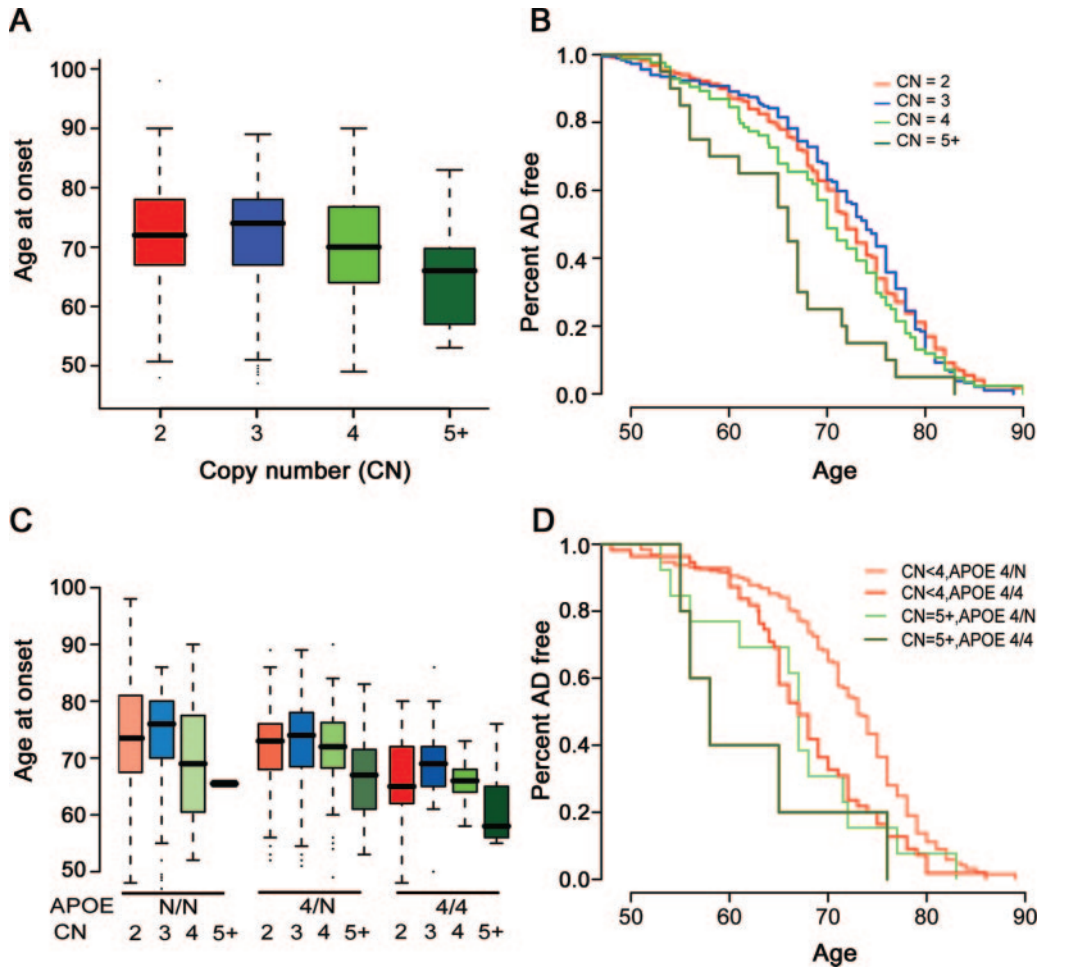
The inclusion criteria aimed to capture a broad range of AAO to increase power which potentially resulted in enrollment of early-onset familial AD cases. We did not screen the cohort for mutations in *APP*, *PSEN1*, and *PSEN2* as cohort studies have demonstrated that mutations in these genes are seldom detected in patients who do not have an autosomal dominant family history of AD. However, in order to further confirm the association, we reanalyzed the subset of the replication cohort with AAO over 60. The olfactory receptor locus remained associated with AAO in the categorical model (RR = 1.72, CI 1.01–2.96, and $p = 0.049$) in the reduced dataset (n = 439).

In the Affymetrix set (n = 243) we used the complete SNP dataset ascertained from the Genome-Wide Human SNP Array 6.0 (Affymetrix) to assess the possibility of population substructure confounding CN state. The population substructure analysis was performed by principal component analysis with the Eigenstrat package for the first 10 principal components.[16] The projected values for the samples were then plotted against each other for the first 2 components (figure e-3).

DISCUSSION AD is one of the most significant public health problems and likely to increase in its burden to society, considering projected increases in longevity and number of aging individuals.[1] Delay of AAO of AD could result in marked decrease in prevalence and therefore AAO is an important attribute of disease and a desired therapeutic target. Although AAO can only be estimated, the usefulness of determining symptom onset via the single question inquiry method has been demonstrated by the identification of the influence of the *APOE*4 allele on AAO.[17] We estimated AAO by 2 methods, the single question inquiry and a validated structured interview with landmark event to facilitate recall of the surrogate historian.[13] The Spearman correlation of the 2 methodologies was significant (Spearman correlation rho = 0.9409, $p = 2.2 \times 10^{-16}$; CI 0.90–0.97) and outlier and residual analysis showed no systematic deviation between the 2 methods.

We have undertaken a cases-only CN variation genome-wide association study with AAO of AD and have detected association of gene dosage of the olfactory receptor region on chromosome 14q11.2 with AAO of AD. The replication cohort confirmed the association. Moreover, this analysis showed the CN association to be independent of and in addition to the association observed for *APOE*4. The CNV contributing to the association signal harbors an olfac-

Figure 3 Variation_0316 association with age at onset (AAO) of Alzheimer disease (AD) in the replication cohort



(A) Box plots depict the dosage association of the inferred copy number variant (CNV) variation_0316 (http://projects.tcag.ca/variation/) with AAO. There is an association of increased CN state with earlier AAO, the largest association signal emerging from the CN state 5+. (B) The corresponding survivorship curves for each of the groups depicted in the box plots. Subsequently, Cox proportional hazard regression was performed using the inferred CNV variation_0316 incorporating *APOE* and gender into the model. (C) Box plots show the dosage association with AAO, this time separating against the various *APOE* backgrounds. In each *APOE* class increased CN state is associated with earlier AAO, again the largest signal emerging from the CN state 5+. The time to event curves in (D) contrast the CN2 and CN3 states (red) with the CN5+ states (green) on the *APOE* N/4 (light) and *APOE* 4/4 (dark) backgrounds.

tory receptor gene cluster, which contains plausible candidate modifier genes in disease pathomechanism in agreement with clinical and neuropathologic observations. The allele frequencies of the CNV region identified are comparable to prior reports,[18] and the allele frequencies in the replication study are consistent with detection of the association between CN and AAO in the discovery cohort. Applying a categorical model to the replication dataset yielded a more significant association, suggesting that the 5+ CN state confers the highest risk.

Although we detected all CN states in the controls as well (table e-5), the sample size is small and the study is not designed for case-control association. In fact, our study design is statistically powerful because the cases-only approach eliminated the misclassification bias in this late-onset disease with age-dependent penetrance. However, the cases-only design is a limitation of our study. Sample size calculations for a case-control association based on an allele frequency of 0.04 (the observed frequency of CN5+ high risk state) and using an estimated odds ratio (OR) of 1.5 (effect size for the CN5+ state) suggest that a sample size of 1,800 cases and 1,800 controls are required to achieve a power of 0.8 to detect an association. This sample size is beyond the scope of this study.

Previous studies[19-21] investigating the genetics of AAO of AD used SNP datasets, and thus our CNV approach is complementary to these studies. The prior SNP association studies are likely to have lacked sensitivity to detect this locus because of its

complex allelic structure. While the ability of SNPs to tag CNVs is debated,[22] loci with multiple types of alleles with rare individual allele frequencies are likely less amenable to SNP tagging. We performed the Cox proportional hazard regression on 148 SNPs flanking the CNV locus using additive, recessive, and dominant models with the assumption that if SNPs are in linkage disequilibrium (LD) with the CNV we would detect the association (table e-6 and figure e-4). Only one SNP (rs11849055) reached Bonferroni-corrected significance in the recessive model, and this SNP is not in LD with the CNV we detected. Our results may suggest that the observed CNV events likely occurred on various SNP haplotypes as independent events.

This study shows an association of CN variation_0316 and AAO, and does not lead us to causal genes per se. However, the association signal arising from a multiallelic CNV locus and the gene dosage association suggest that the gene content of the CNV including the olfactory receptor cluster is a potential modifier of AAO in AD. Prospective cohort studies established the risk olfactory deficit confers for the development of cognitive decline. A prospective study of 1,920 cognitively normal subjects found a significant association between olfactory impairment at baseline and 5-year incidence of cognitive impairment (OR = 6.62, CI = 4.36–10.05).[23] Another study of 1,604 older adults without dementia found that women with anosmia who possessed at least one *APOE4* allele had an OR of 9.71 for development of cognitive decline over the ensuing 2 years, compared with an OR of 1.9 for women with no olfactory dysfunction and at least one such allele.[24] Neuropathologic series have found marked cell loss in the olfactory bulb and anterior olfactory nucleus and the presence of disease-related pathology: neuritic plaques and neurofibrillary tangles in these structures.[25] Whether loss of olfaction is a primary or secondary phenomenon in the pathomechanism of AD is unclear; however, this genetic observation raises the possibility for its role in modifying the AAO.

Although the allele frequency is low, in a disease with a prevalence of 5.3 million a frequency of 4% translates to over 200,000 subjects with the high CN state affected by the disease. Patients with AD with the earliest AAO represent the most desired group for intervention with disease-modifying therapy. Pathogenic CNVs may be more amenable to therapy than other types of genetic variation. In contrast to mutation events resulting in loss of function or toxic gain of function, CNVs alter gene dosage, allowing modification by small molecules which may offset the dosage effects.[26] For example, the CMT1A duplication rat model treated with a progesterone antagonist achieved clinical and pathologic improvement even by partial correction of gene expression by epigenetic modification.[27] If confirmed, evaluation of olfactory receptor CN may lead to both earlier detection and potential disease-modifying therapy, both of which could have a major impact on the public health burden of AD.

AUTHOR CONTRIBUTIONS

Statistical analysis was conducted by Dr. C.A. Shaw.

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DISCLOSURE

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