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Neurology 2012;78;1250; Published online before print April 4, 2012;
DOI 10.1212/WNL.0b013e3182515972

Role of common and rare *APP* DNA sequence variants in Alzheimer disease

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ABSTRACT

Objectives: More than 30 different rare mutations, including copy number variants (CNVs), in the amyloid precursor protein gene (*APP*) cause early-onset familial Alzheimer disease (EOFAD), whereas the contribution of common *APP* variants to disease risk remains controversial. In this study we systematically assessed the role of both rare and common *APP* DNA variants in Alzheimer disease (AD) families.

Methods: Families with EOFAD genetically linked to the *APP* region were screened for missense mutations and locus duplications of *APP*. Further, using genome-wide DNA microarray data, we examined the *APP* locus for CNVs in a total of 797 additional early- and late-onset AD pedigrees. Finally, 423 single nucleotide polymorphisms (SNPs) in the *APP* locus, including 2 promoter polymorphisms previously associated with AD risk, were tested in up to 4,200 individuals from multiplex AD families.

Results: Analyses of 8 21q21-linked families revealed one family carrying a nonsynonymous mutation in exon 17 (Val717Leu) and another family with a partially penetrant 3.5-Mb locus duplication encompassing *APP*. CNV analysis in the *APP* locus revealed an additional family carrying a fully penetrant 380-kb duplication, merely spanning *APP*. Last, contrary to previous reports, association analyses of more than 400 different SNPs in or near *APP* failed to show significant effects on AD risk.

Conclusion: Our study shows that *APP* mutations and locus duplications are a very rare cause of EOFAD and that the contribution of common *APP* variants to AD susceptibility is insignificant. Furthermore, duplications of *APP* may not be fully penetrant, possibly indicating the existence of hitherto unknown protective genetic factors. ***Neurology*® 2012;78:1250–1257**

GLOSSARY

Aβ = amyloid-β; **AD** = Alzheimer disease; **APP** = amyloid precursor protein; **CNV** = copy number variant; **EOFAD** = early-onset familial Alzheimer disease; **FISH** = fluorescent in situ hybridization; **GWAS** = genome-wide association study; **LOAD** = late-onset Alzheimer disease; **NCRAD** = National Cell Repository for Alzheimer's Disease; **NIA** = National Institute on Aging; **NIMH** = National Institute of Mental Health; **SNP** = single nucleotide polymorphism.

Highly penetrant mutations in the gene encoding amyloid precursor protein (*APP*) (21q21.2) were the first reported genetic causes of early-onset familial Alzheimer disease (EOFAD)[1] (see also Alzheimer Disease & Frontotemporal Dementia Mutation Database, www.molgen.ua.ac.be/ADMutations/[2]). Most of the currently known AD-causing mutations in *APP* lead to an increase in the ratio of the amyloid-β_{42} ($A\beta_{42}$) to $A\beta_{40}$ peptide[3,4] and synaptic Aβ levels.[5] AD pathology is also found in patients with Down syndrome, i.e., trisomy of chromosome 21, indicating that extra copies of *APP* alone may lead to neurotoxic Aβ production in the absence of any missense mutations. Furthermore, several reports have shown that the presence of *APP* locus duplications cause EOFAD.[6–10] Finally, recent candidate gene studies have also implicated the existence of rare variants in the *APP* promoter in EOFAD by increasing *APP* expres-

Supplemental data at www.neurology.org

Supplemental Data

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Study funding: Funded by the Cure Alzheimer Fund (to L.B. and R.E.T.) as part of the "Alzheimer Genome Project™" and by grants from the NIA (5R01-AG23667 to L.B.) and the NIMH (5R37-MH60009 to R.E.T.).

Disclosure: Author disclosures are provided at the end of the article.

Table 1 Genetic association results of 2 *APP* promoter polymorphisms previously associated with AD risk[a]

	NIMH		NCRAD		NIA		CAG		Combined	
SNP	*p* value	Fams	*p* value	Fams	*p* value	Fams	*p* value	Fams	*p* value	Fams
rs463946 [′−3102G/C]	0.79	21	0.28	19	0.25	20	0.65	9	0.13	69
rs459543 [+37C/G]	0.91	19	0.74	15	0.22	18	−0.91	9	0.41	61

Abbreviations: AD = Alzheimer disease; APP = amyloid precursor protein; CAG = Consortium on Alzheimer's Genetics; NCRAD = National Cell Repository for Alzheimer's Disease; NIA = National Institute on Aging; NIMH = National Institute of Mental Health; SNP = single nucleotide polymorphism.

[a] *p* Value represents *p* values calculated using PBAT with additive model and affection status, restricted to Caucasian-only families (negative *p* values indicate undertransmission to affected family members). Fams represents the number of informative families. Combined represents meta-analysis of results across all 4 samples using METAL. See table e-2 for meta-analysis results on 421 genotyped and imputed SNPs in the *APP* region determined in the NIMH and NCRAD samples.

sion,[11,12] although these findings have been refuted elsewhere.[13–19] In contrast, the contribution of common *APP* variants to Alzheimer disease (AD) risk remains unclear (see also AlzGene database, www.alzgene.org[20]). In this study, we thoroughly investigated the role of both rare and common *APP* DNA sequence variants in several large collections of both EOFAD and late-onset AD (LOAD) families. Our results suggest that missense mutations in *APP* and locus duplications are a rare cause of AD, whereas common variants in *APP* probably play no major role, if any, in modulating AD risk. In addition, we observe evidence that some *APP* locus duplications may only display reduced penetrance.

METHODS

Participants. ***National Institute of Mental Health families.*** In total, this sample includes 1,536 individuals from 457 multiplex AD families.[21] Of these, 131 pedigrees (517 subjects [316 affected subjects, onset age 64.5 + 9.5 years]) are from families with an "early/mixed" onset age, i.e., at least one sampled affected subject showed an onset age of <65 years), whereas in the remaining pedigrees all sampled affected subjects showed an onset age of ≥65 years. Age at onset for all cases of AD was determined by a clinician based on an interview with a knowledgeable informant and review of any available records. From our earlier whole-genome linkage screen on these families,[22] we identified 8 families in the early/mixed onset-age stratum that showed evidence of genetic linkage to the region encompassing *APP* at ~26 Mb (i.e., between markers D21S1437 at ~20 Mb and D21S1440 at ~38 Mb) (table 1).

Additional independent family samples. In addition to the National Institute of Mental Health (NIMH) families, we analyzed members of 3 independent AD family collections. Two of these were obtained from the National Cell Repository for Alzheimer Disease (NCRAD), and ascertainment and collection details can be found at the NCRAD Web site (www.ncrad.org). The collection of families labeled here as NIA (National Institute on Aging) comprised 1,111 samples from 351 pedigrees (Caucasian: 1,040 samples from 329 pedigrees). The collection of families labeled here as NCRAD comprised 1,260 samples from 368 pedigrees (Caucasian: 1,106 samples from 330 pedigrees). Finally, the collection of families labeled CAG (Consortium on Alzheimer's Genetics) originated from multiple NIA-funded Alzheimer Disease Research Centers under the auspices of the Consortium on Alzheimer's Genetics. Probands were included only if they had at least one unaffected living sibling willing to participate in this study. For all non-NIMH families we only included pedigrees in which all sampled affected individuals had onset ages of at least 50 years.

Note that different combinations of these family samples were used in different parts of our study. *APP* sequencing was performed in chromosome 21–linked NIMH families only. *APP* copy number variant (CNV) and common marker association analyses were performed on all remaining NIMH and all the NCRAD families. Last, members from all 4 family samples (i.e., 4,180 individuals) were genotyped for the 2 previously associated *APP* promoter single nucleotide polymorphisms (SNPs) (rs459543 [+37c/g] and rs463946 [−3102G/C]).

Standard protocol approvals, registrations, and patient consents. Written informed consent for participation was provided by all subjects or, for those with substantial cognitive impairment, by a caregiver, legal guardian, or other proxy by the clinical sites responsible for subject recruitment. The study protocols for all populations were reviewed and approved by the institutional review boards of the respective recruitment sites. Genetic experiments were reviewed and approved by the institutional review board of Massachusetts General Hospital.

Experimental procedures. ***Fluorescent in situ hybridization.*** Bacterial artificial chromosome clones containing both ends of the *APP* gene (RP11-15D13 for the 5′ end and RP11-410J1 for the 3′ end) and CTB-63H24 mapping to 21q22.3 (control probe) were used for fluorescent in situ hybridization (FISH). RP11-15D13 and RP11-410J1 were labeled with Cy3-dUTP, and CTB-63H24 was labeled with fluorescein isothiocyanate-dUTP by nick translation. FISH was performed according to the protocol described in Mohapatra et al.[23]

SNP genotyping. Genome-wide association study (GWAS) SNPs were generated in a separate project (L. Bertram, A.R. Parrado, B. Hooli, C. Lange, R.E. Tanzi, 2012, unpublished) on the Affymetrix Genome-Wide Human SNP Array 6.0, using individually optimized genotyping and allele-calling procedures. Before statistical analyses, the number of SNPs was augmented by genotype imputation using

IMPUTE2.0 software. Details on the methods used for genotype imputation and statistical analyses can be found in appendix e-1 on the *Neurology*® Web site at www.neurology.org.[24–27] The 2 promoter SNPs (rs459543 [+37c/g] and rs463946 [−3102G/C]) were assayed separately via high-efficiency fluorescence polarization-detected single base extension on a Criterion Analyst AD high-throughput fluorescence detection system (Molecular Devices), using customized PCR primers and cycling conditions.[28] Genotyping efficiency on the 2 SNPs tested here was >95%, whereas the error rate was <1% (based on ~10% duplicated samples). Both SNPs were in Hardy-Weinberg equilibrium ($p > 0.05$) in all 4 samples.

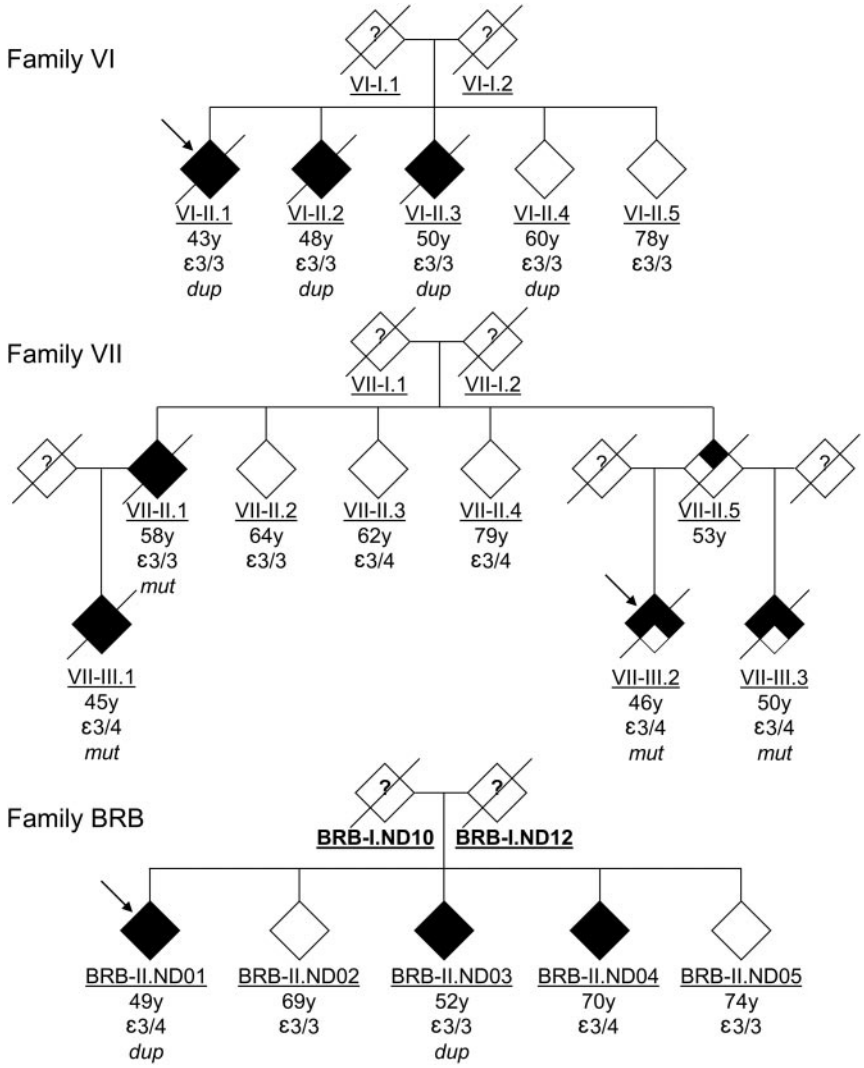
CNV analysis. This was done for individuals from the NIMH and NCRAD datasets for whom we used the Affymetrix Genome-Wide Human SNP Array 6.0 as part of an ongoing GWAS. Raw probe intensities from each sample were normalized against the HapMap CEU reference intensity dataset. For the purpose of CNV analysis, we excluded samples showing chromosomal abnormalities and high CNV count. All samples included were subject to waviness factor adjustment.[29,30] CNV calling and segmentation were performed with PennCNV (www.openbioinformatics.org/penncnv/[30]) using default criteria.

Fluidigm Digital Array protocol. We used this method as a validation experiment for the *APP* duplication observed in the NCRAD family. In brief, 16 ng of DNA from all subjects was mixed with 1× TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA), 1× FAM-labeled *APP* copy number probe (Hs05532959_cn, Applied Biosystems), 1× VIC-labeled RNase P TaqMan assay, and 1× sample loading reagent (Fluidigm Inc., South San Francisco, CA). DNA samples from individuals of NIMH family VI carrying an *APP* duplication were used as positive controls, and all samples were run on a 48.776 array. The experimental protocol and data analysis procedures are described in detail elsewhere.[31,32]

RESULTS The 8 EOFAD (table 1) families showing linkage at or near the *APP* region were resequenced for mutations in *APP* exons 16 and 17, the promoter region (−1 to −676 kb), and the 3′ untranslated region (1–1,221 bp). In addition, these families were also tested for *APP* locus duplications using semiquantitative multiplex PCR and subsequently confirmed by FISH (figure e-1). One family (VII) carried a previously reported and fully penetrant missense mutation in exon 17 (Val717Leu, rs63750264 G>C, "Indiana-2"[33]) (figure 1). The age of onset of AD in this family was between 45 and 58 years, whereas the 3 unaffected individuals were between 62 and 79 years of age at last examination. The clinical diagnosis of AD was confirmed neuropathologically in 2 affected individuals of this pedigree (VII-II.3 and VII-II.1), whereas the other 2 received a diagnosis of probable AD (VII-II.2 and VII-II.4). A second chromosome 21–linked family (VI) (figure 1) was found to carry *APP* locus duplication which, in contrast to previous reports, only showed partial penetrance. Using genome-wide microarray data we were able to delineate the size of the duplicated segment to ~3.4 Mb (figure 2). In this family, the duplication was present in all 3 affected individuals (onset ages: 43–50 years; all AD diagnoses confirmed by neuropathologic examination), but also in one unaffected individual (VI-II.4, last age at examination 60 years), whereas no duplication was found in the remaining unaffected sibling (VI-II.5, 78 years) (figure 1). Tests for expression level differences of *APP* mRNA and protein, as well as Aβ levels, in Epstein-Barr virus–transformed lymphoblastoid cell lines of all members of this family did not show significant differences between carriers and noncarriers of the *APP* duplication, regardless of affection status (data not shown). This finding is in line with earlier reports indicating that pathologically relevant increases in *APP*/Aβ expression may be restricted to the brain and are not detectable in peripheral cells.[12] Unfortunately, brain samples were not available for any member of this family.

Analysis for CNVs in *APP* in the microarray data from the remaining 429 NIMH families and 368 NCRAD families revealed one family in the NCRAD dataset (BRB, figure 1) carrying *APP* locus duplication, which was subsequently confirmed using the Fluidigm Digital Array (table e-1). Although fully penetrant, this latter duplication is interesting for 2 reasons. First, the duplicated segment (~0.38 Mb) is approximately 10-fold smaller than the duplicated segment identified in the NIMH family. Barely encompassing the entire genomic interval of *APP*, this segment represents the shortest *APP* duplication identified to date (figure 2). Second, the duplication was carried by only 2 of 3 affected siblings in this family (onset ages 49 and 52 years), whereas a third affected individual (onset age 70 years) showed a diploid, i.e., normal, copy status in this region. Similarly, the 2 unaffected siblings (ages at last examination 69 and 74 years) also showed no evidence for duplication of the *APP* region. Thus, this family coalesces the sort of genetic heterogeneity that is typical of AD (and several other neurodegenerative disorders), i.e., the presence of likely disease-causing and susceptibility-increasing factors.

Figure 1 Pedigree charts of families found to carry disease-causing *APP* mutations and locus duplications

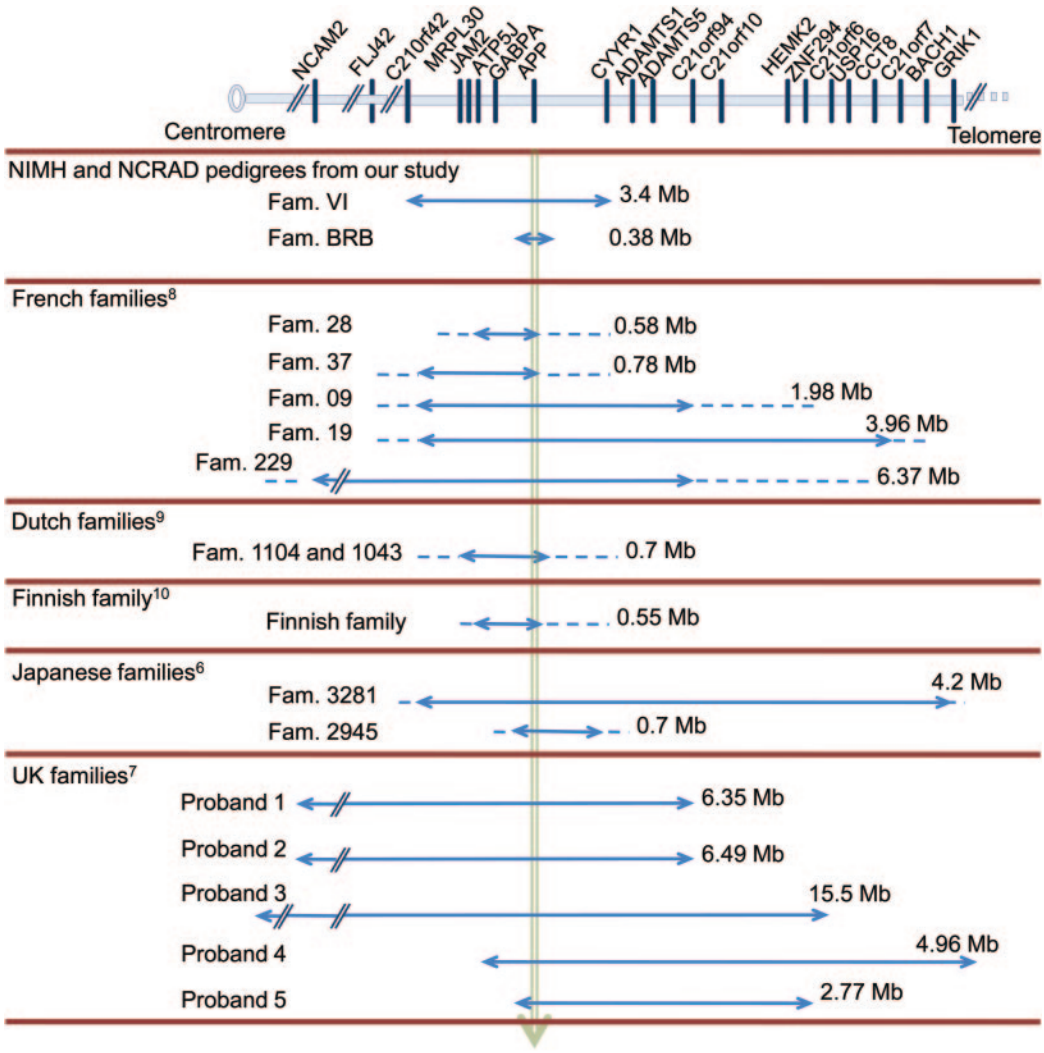


Information for each individual is (from top to bottom): age at onset (in affected individuals) or age at last examination (unaffected individuals); *APOE* genotype; and *APP* mutation finding. Probands are indicated by arrows. No DNA or clinical information was available from the founders (?). dup = carriers of *APP* duplication; mut = carriers of Val717Leu mutation.

Finally, association analyses of 421 common SNPs located within a 340-kb interval encompassing *APP* was undertaken based on observed and imputed GWAS microarray SNP data probing for effects on disease risk. However, the strongest association with AD risk in these analyses only showed a nominal p value of ~0.008 (rs117650267) (table e-2), which would not even approach statistical significance if corrected for multiple testing. Given our relatively liberal choice of including imputed genotypes (based on a posterior call probability of 0.5), it is unlikely, albeit not impossible, that the use of overstringent quality control procedures precluded us from detecting any genuine association results, e.g., by excluding certain informative SNPs or families. We did fail to detect evidence for association with 2 SNPs in the *APP* promoter region (rs459543 [+37C/G] and rs463946 [−3102G/C]) (table 1) previously reported to be associated with AD risk.[13,14] Because these SNPs were not observed or imputed by the Affymetrix microarray, they were manually genotyped in nearly 4,200 individuals originating from 4 independent family datasets, yielding >70% power to detect the previously reported effect sizes. The absence of significant association using common polymorphisms is in line with recent GWASs reporting no evidence of association with markers near the *APP* region (see the AlzGene database for a list of all GWASs performed in AD).

DISCUSSION We undertook a systematic assessment of the contribution of rare and common *APP* DNA sequence variants across large collections of independent AD family samples. Mutational screening

Figure 2 Delineation of the *APP* duplicated region identified here and those in previous studies



Approximate locations of the duplicated intervals across studies are shown. Solid arrows indicate minimal size of the duplicated interval; dotted lines indicate maximal boundaries. Note that our study is the only to use high-density genome-wide association study data, allowing a much more precise delineation of the duplicated interval than the lower-resolution microsatellite-based mapping. Physical location of duplicated segments from microarray data mapped to hg18 assembly are family (Fam.) VI (chr21: 23984747–27466529) and family BRB (chr21: 26125668–26505191).

of EOFAD families linked to the *APP*-encompassing region on chromosome 21 revealed one family carrying a previously reported missense mutation and one family carrying a duplication of the *APP* locus. Although the missense mutation showed complete penetrance in the affected family, the occurrence of one unaffected individual in the family VI carrying the *APP* duplication at >3 SD from the average familial onset age is strongly indicative of incomplete penetrance, implying existence of yet unidentified protective factors. Although the possibility that this individual will also develop AD at some later time cannot be definitely excluded, our findings already suggest that other genetic or nongenetic factors can mitigate the effects of *APP* locus duplications and either confer complete protection against AD or at least substantially delay its onset age. In the currently available literature there is one other report in which an unaffected individual was also found to carry an *APP* duplication (individual III.21 in family 1104[9]). However, the last age of examination of this individual is still within 1 SD of the average familial onset age, whereas the difference here is greater than 3 SD in the unaffected sibling in family VI. The second, independent *APP* duplication observed in our study (family BRB in the NCRAD dataset) represents the smallest reported duplicated interval on chromosome 21, effectively reducing the obligate AD-causing region to *APP* (chr21: 26122781–26521135, NCBI36/hg18 assembly). To date, this is also the first reported case of an *APP* duplication co-occurring with another cause of AD within the same

pedigree. Aggregating the CNV data across different studies published to date (figure 2) suggests that most (if not all) instances of locus duplications in this chromosomal interval are not linked to the same founder individual, but rather have occurred independently of one another. Overall, these results suggest that *APP* duplications are a rare cause of EOFAD and extremely rare (if not absent) in LOAD.

Contrary to these findings confirming and extending prior evidence, we were unable to corroborate the presence of sequence variants in the *APP* promoter, neither as causative nor as risk factors for AD. This includes variants 534G→A, 479C→T, 369C→G, and 118C→A, which were previously shown to cause AD by increasing expression levels of *APP*.[12] None of the NIMH chromosome 21–linked families carried any mutations in the *APP* promoter region, including the variants described above. Our failure to detect mutations at these sites are in agreement with a prior study[14] reporting a higher frequency of the presumed disease-causing alleles in healthy controls compared with individuals with AD. Taken together, these data suggest that these *APP* promoter sequence variants do not have a role in AD pathogenesis. This raises the possibility that other, hitherto elusive, DNA sequence variants in or near *APP* may account for the onset of AD in the 6 families linked to chromosome 21q but not found to carry any disease-causing *APP* mutations (table e-3). Given the current lack of evidence implying AD-causing mutations in regions beyond those investigated here (see Alzheimer Disease & Frontotemporal Dementia Mutation Database), this alternative appears unlikely.

Finally, genetic association analyses of common variants, including 2 *APP* promoter polymorphisms previously reported to show association with LOAD risk, did not reveal any significant evidence for association with either risk for AD or onset-age variation. Whereas some equipoise from earlier and often smaller studies still exists, our results are in line with, and substantially extend, those of a recent study investigating 44 SNPs in almost 1,200 case patients and controls from the United States,[34] although that study did not directly test the 2 previously associated promoter SNPs (rs459543 and rs463946) that were investigated here. In addition, none of the currently published GWASs in AD (see www.alzgene.org for details) have thus far reported significant association between risk for AD and common sequence variants in or near *APP*, providing further evidence against the notion that common sequence variation in this gene contributes to risk for LOAD. Although this is similar to the lack of risk associated with common variants in the other 2 EOFAD genes, *PSEN1* and *PSEN2* (presenilin 1 and 2), it is in contrast to other neurodegenerative disorders, e.g., Parkinson disease or frontotemporal dementia, for which genes known to contain rare, disease-causing variants giving rise to disease forms transmitted in a Mendelian fashion are also among the lead GWAS findings based on common polymorphisms.[35] It remains to be seen whether the investigation of subjects drawn from genetic backgrounds other than Caucasian will reveal different patterns.

Our comprehensive and systematic analyses investigating the role of *APP* in AD genetics in subjects of Caucasian descent suggest that missense mutations in *APP* and locus duplications are a rare cause of AD, whereas common variants in *APP* probably play no major role, if any, in contributing to risk for AD. In addition, the incomplete penetrance of the *APP* locus duplication observed in family VI emphasizes the need to more systematically search for protective variables. A better understanding of these risk-reducing factors may be essential for developing better and more effective early prevention and treatment strategies against this devastating disorder.

AUTHOR CONTRIBUTIONS

Dr. Hooli: acquisition of data, analysis of data, study coordination, drafting and revision. Dr. Mohapatra: acquisition of data, analysis of data and revision. Dr. Mattheisen: statistical analyses. Dr. Parrado: analysis of data. J.T. Roehr: acquisition of data and analysis of data. Dr. Shen: acquisition of data. Dr. Gusella: study supervision and revision. Dr. Moir: study supervision and revision. Dr. Saunders: study supervision and revision. Dr. Lange: study design, revision. Dr. Tanzi: study supervision and coordination, revision. Dr. Bertram: study concept and design, analysis of data, statistical analysis, study supervision and coordination, drafting and revision.

ACKNOWLEDGMENT

The authors thank all families for participating in this study. Data and biomaterials were collected in 3 projects that participated in the NIMH Alzheimer Disease Genetics Initiative. From 1991 through 1998, the principal investigators and co-investigators were the following: Massachusetts General Hospital, Boston, MA (U01-MH46281), Marilyn S. Albert, PhD, and Deborah Blacker, MD, ScD; Johns Hopkins University, Baltimore, MD (U01-MH46290), Susan S. Bassett, PhD, Gary A. Chase, PhD, and Marshal F. Folstein, MD; and University of Alabama, Birmingham, AL (U01-MH46373), Rodney C.P. Go, PhD, and Lindy E. Harrell, MD.

DISCLOSURE

Dr. Hooli, Dr. Mohapatra, Dr. Mattheisen, Dr. Parrado, and J. Roehr report no disclosures. Dr. Shen receives research support from Children's Tumor Foundation. Dr. Gusella serves on a scientific advisory board and as a consultant for Quest Diagnostics, Inc.; serves on editorial advisory boards for *DNA and Cell Biology*, *Neurobiology of Disease*, *Neurogenetics*, *Biomed Central Neuroscience*, *Biomed Central Biology*, and *Molecular Autism*; holds patents re: Methods for altering mRNA splicing and treating familial dysautonomia and other mechanistically related disorders; Methods for detecting mutations associated with familial dysautonomia; Gene for identifying individuals with familial dysautonomia; Vectors for delivering viral and oncogenic inhibitors; Kits for detecting polymorphisms associated with familial dysautonomia; Tumor suppressor merlin and antibodies thereof; Use of genetic markers to diagnose familial dysautonomia; TPR-containing genes; Tumor suppressor gene merlin; Huntingtin

DNA, protein and uses thereof; Transport protein gene from the Huntington's disease region; Flexible scheme for admission control of multimedia streams on integrated networks; Use of genetic markers to diagnose familial dysautonomia; DNA encoding a protein-coupled receptor kinase; Test for Huntington's disease; and Isolation and localization of DNA segments; and receives/has received research support from the NIH (NINDS, NIGMS, NICHD), the Simons Foundation Autism Research Initiative, CHDI Foundation Inc., Autism Speaks, and the Huntington's Disease Society of America Coalition for the Cure. Dr. Moir serves as an Assistant Editor for the *International Journal of Genetic and Medical Nanotechnology*; holds a patent re: A novel method to diagnose and/or treat Alzheimer's disease; and receives research support from the Helmsley Foundation, the NIH, and the Cure Alzheimer's Fund. Dr. Saunders receives research support from the NIH/NINDS and the Cure Alzheimer's Fund. Dr. Lange reports no disclosures. Dr. Tanzi serves as a consultant for and has received funding for travel and speaker honoraria from Pfizer Inc, Eisai Inc., Prana Biotechnology, and Probiodrug AG; serves on the editorial board of *Neuron* editorial board; and receives research support from the NIH/NIMH and the Cure Alzheimer's Fund. Dr. Bertram serves on editorial advisor boards for *Neurogenetics*, *European Journal of Clinical Investigation*, and *International Journal of Molecular Epidemiology and Genetics*.

Received October 5, 2011. Accepted in final form November 28, 2011.

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