

ORIGINAL ARTICLE

## Family-Based Association between Alzheimer's Disease and Variants in *UBQLN1*

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### ABSTRACT

#### BACKGROUND

Recent analyses suggest that the known Alzheimer's disease genes account for less than half the genetic variance in this disease. The gene encoding ubiquilin 1 (*UBQLN1*) is one of several candidate genes for Alzheimer's disease located near a well-established linkage peak on chromosome 9q22.

#### METHODS

We evaluated 19 single-nucleotide polymorphisms in three genes within the chromosome 9q linkage region in 437 multiplex families with Alzheimer's disease from the National Institute of Mental Health (NIMH) sample (1439 subjects). We then tested the single-nucleotide polymorphisms showing a positive result in an independently identified set of 217 sibships discordant for Alzheimer's disease (Consortium on Alzheimer's Genetics [CAG] sample; 489 subjects) and assessed the functional effect of an implicated single-nucleotide polymorphism in brain tissue from 25 patients with Alzheimer's disease and 17 controls.

#### RESULTS

In the NIMH sample, we observed a significant association between Alzheimer's disease and various single-nucleotide polymorphisms in *UBQLN1*. We confirmed these associations in the CAG sample. The risk-conferring haplotype in both samples was defined by a single intronic single-nucleotide polymorphism located downstream of exon 8. The risk allele was associated with a dose-dependent increase in an alternatively spliced *UBQLN1* (lacking exon 8) transcript in RNA extracted from brain samples of patients with Alzheimer's disease.

#### CONCLUSIONS

Our findings suggest that genetic variants in *UBQLN1* on chromosome 9q22 substantially increase the risk of Alzheimer's disease, possibly by influencing alternative splicing of this gene in the brain.

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**A**LZHEIMER'S DISEASE IS THE MOST common form of age-related dementia and one of the most serious health problems in the industrialized world. Genetically, Alzheimer's disease is heterogeneous and complex, displaying an age-dependent dichotomy.<sup>1</sup> To date, more than 160 highly penetrant but rare mutations have been described in three genes (*APP*, *PSEN1*, and *PSEN2*) that cause early-onset familial Alzheimer's disease. With respect to the more common, late-onset form of Alzheimer's disease, a polymorphism in the apolipoprotein E gene (*APOE*  $\epsilon$ 4) has been associated with increased susceptibility.<sup>2,3</sup> However, these four genes together may account for less than half the genetic variance in Alzheimer's disease, and possibly up to seven additional Alzheimer's disease genes remain to be identified.<sup>4</sup>

In an attempt to pinpoint these additional Alzheimer's disease loci, we recently completed a high-resolution, full-genome screen using a large, uniformly ascertained and evaluated cohort of families with Alzheimer's disease, the National Institutes of Mental Health (NIMH) Genetics Initiative Alzheimer's disease study sample.<sup>5</sup> One of the regions with the strongest evidence of linkage in that study is located on chromosome 9q22, 99 cM (90 Mb) from the tip of the short arm (Fig. 1). We also observed a smaller linkage peak on 9p21, approximately 55 cM (30 Mb) from the tip of the short arm. Several independent studies<sup>6</sup> have provided additional evidence linking Alzheimer's disease to both these regions.

To characterize the putative Alzheimer's disease locus or loci on chromosome 9q, we used a positional candidate-gene strategy involving family-based genetic-association analyses of three genes (*APBA1*, *UBQLN1*, and *ABCA1*) in two independent family-based cohorts with Alzheimer's disease. These genes were chosen on the basis of their proven or suggested functional involvement in the pathogenesis of Alzheimer's disease. To our knowledge, of these, only *ABCA1* has previously been tested for a genetic association with Alzheimer's disease.<sup>7</sup>

## METHODS

### SUBJECTS AND SAMPLES

All subjects or, in those with substantial cognitive impairment, a caregiver, legal guardian, or other proxy gave written informed consent for participation in this study. The study protocols for both samples were reviewed and approved by the institutional review board of Partners HealthCare System.

### *The NIMH Genetics Initiative Alzheimer's Disease Study Sample*

Subjects were identified and enrolled in the study cohort from January 1991 to September 1997 according to a standardized protocol applying the criteria of the National Institute of Neurological and Communicative Diseases and Stroke/Alzheimer's Disease and Related Disorders Association (NINCDS/ADRDA)<sup>8</sup> for the diagnosis of Alzheimer's disease.<sup>9</sup> During the 10 or so years that the participating families have been followed, a clinical diagnosis of Alzheimer's disease has been confirmed at autopsy in 94 percent of those identified as having Alzheimer's disease.<sup>5</sup> The full NIMH sample includes 1439 subjects (69 percent of whom are women) from 437 families with at least 2 affected family members: 994 subjects with Alzheimer's disease (mean [ $\pm$ SD] age at onset, 72.4 $\pm$ 7.7 years; range, 50 to 97), 411 unaffected subjects, and 34 subjects with an unknown phenotype (additional details are provided in the Supplementary Appendix, available with the full text of this article at [www.nejm.org](http://www.nejm.org)).

### *Consortium on Alzheimer's Genetics Study Sample*

Subjects for the second, independently ascertained cohort of families with Alzheimer's disease were identified and enrolled under the auspices of the Consortium on Alzheimer's Genetics (CAG) study (see the Supplementary Appendix). NINCDS/ADRDA criteria were used to make a clinical diagnosis of Alzheimer's disease,<sup>8</sup> and probands were included only if they had at least one unaffected living sibling willing to participate in the study. Unlike the NIMH sample, no affected family member other than the proband was required; thus, the majority of families included only one subject with Alzheimer's disease. Most sibships consisted of just one discordant pair of siblings, but 41 families included more than two siblings (see the Supplementary Appendix). Data and specimen collection began in October 1999 (and is ongoing) and has been completed for 489 subjects (63 percent of whom are women) from 217 sibships: 224 affected subjects who were at least 50 years of age at the onset of Alzheimer's disease (mean age at onset, 71.2 $\pm$ 9.1 years; range, 50 to 89) and 265 unaffected subjects.

### *Brain Specimens*

Temporal neocortical tissue from the brains of 25 patients with Alzheimer's disease and 17 control subjects was used for this study. All patients with Alzheimer's disease had been evaluated at the

Memory Disorders Unit at Massachusetts General Hospital and met both clinical diagnostic criteria (NINCDS/ADDA)<sup>8</sup> and neuropathological diagnostic criteria (Consortium to Establish a Registry for Alzheimer Disease, the National Institute on Aging, and the Reagan Institute Working Group),<sup>10,11</sup> as described previously.<sup>12</sup> Thirteen of the control subjects, defined as not having had any history or neuropathological signs of a brain disorder, had also undergone autopsy at Massachusetts General Hospital; tissue from the other four controls was from the Harvard Brain Tissue Resource Center or the University of Maryland. Among the patients with Alzheimer's disease, 56 percent were men, the average ( $\pm$ SE) age at death was  $81.0 \pm 1.5$  years, and brain specimens were obtained an average of  $13.3 \pm 1.6$  hours after death. Among the controls, 47 percent were men, the average age at death was  $81.6 \pm 2.3$  years, and specimens were obtained an average of  $20.8 \pm 3.6$  hours after death.

#### MICROSATELLITE GENOTYPING

Marker genotypes for the full genome screen were provided by the Center for Inherited Disease Research; genotyping protocols can be found at [www.cidr.jhmi.edu](http://www.cidr.jhmi.edu). The average spacing between markers within the linked interval (e.g., between markers D9S925 and D9S930 in Fig. 1A) was 8.8 cM. For this study, we added markers D9S1800 (average heterozygosity, 0.67) and D9S280 (average heterozygosity, 0.70), narrowing the average marker spacing in this interval to 7.3 cM. Genotyping was done in a 96-well format by means of a polymerase-chain-reaction (PCR) assay followed by amplicon separation with the use of capillary electrophoresis (MegaBACE-1000, Amersham Biosciences) according to the manufacturer's recommendations (see the Supplementary Appendix).

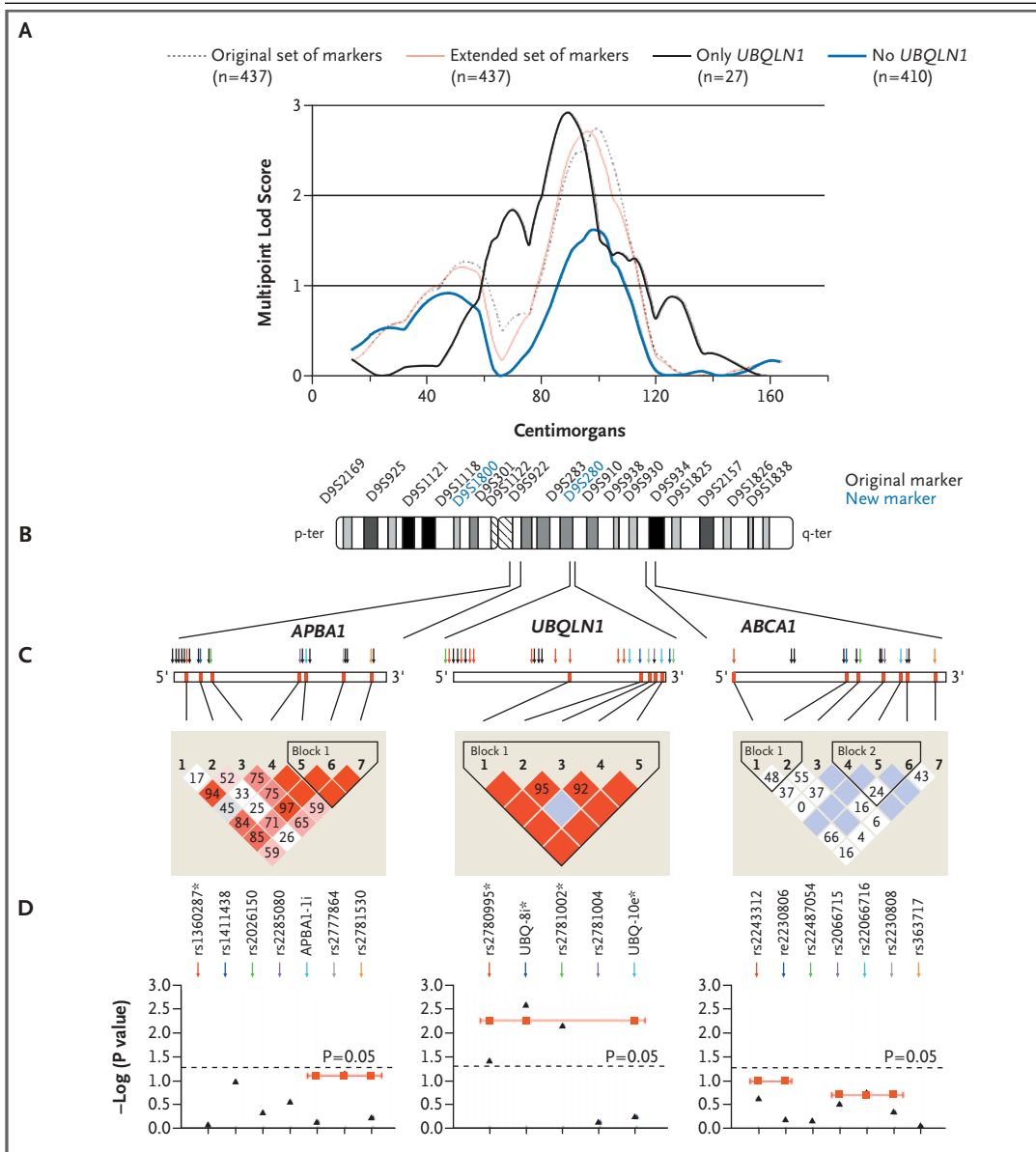
#### IDENTIFICATION AND GENOTYPING OF SINGLE-NUCLEOTIDE POLYMORPHISMS

A total of 58 single-nucleotide polymorphisms were identified in the three candidate genes for Alzheimer's disease either by direct sequencing of DNA from affected and unaffected members of linked families (i.e., those with the highest identity-by-descent allele-sharing statistics) or from publicly available databases (see the Supplementary Appendix). Single-nucleotide-polymorphism genotypes were generated with the use of the fluorescence polarization-detected single-base extension method on a Criterion Analyst AD high-throughput fluo-

#### Figure 1 (facing page). Gene-Mapping Strategy to Identify the Putative Locus for Alzheimer's Disease on Chromosome 9q22.

Panel A shows the results of genetic multipoint linkage analyses involving GeneHunter-Plus software in the full NIMH cohort in the original genome screen and in extended analyses. Numbers in parentheses are the numbers of families analyzed. Panel B shows a cytogenetic ideogram of chromosome 9 with the approximate locations of the candidate genes for Alzheimer's disease: *APBA1*, *UBQLN1*, and *ABCA1*. Panel C shows all single-nucleotide polymorphisms assessed in the candidate genes. Black arrows indicate variants that were either not polymorphic or not amenable to genotyping; arrows of the same color represent single-nucleotide polymorphisms in complete linkage disequilibrium. Below the single-nucleotide polymorphisms are the haplotype blocks as determined with the use of Haploview software on the basis of the strength of the linkage disequilibrium. Colored diamonds indicate pairwise linkage disequilibrium between all markers that were genotyped in the NIMH families. Shades of red and white represent decreasing degrees of linkage disequilibrium between pairs of single-nucleotide polymorphisms, blue strong linkage disequilibrium with a low level of significance, and gray weak linkage disequilibrium with a high level of significance (for details on linkage-disequilibrium measures used in *UBQLN1*, see Table 7 in the Supplementary Appendix). For *UBQLN1* and *APBA1*, these calculations are based on genotypes in all NIMH families, whereas for *ABCA1*, only families in the screening sample were included (see the Methods section in the Supplementary Appendix), resulting in overall lower significance values. Panel D shows the results of single-locus and haplotype analyses with the use of FBAT software (only the NIMH screening sample was included in the analysis of *ABCA1*). Triangles represent single-locus results, and squares represent haplotype-based results (connected by red lines) with the use of haplotype-tagging single-nucleotide polymorphisms according to the predicted haplotype block. Dotted lines indicate a nominal P value of 0.05. Asterisks represent results after combining genotypes from the NIMH and CAG samples (Table 1; see Tables 1 and 2 in the Supplementary Appendix).

rescence detection system (Molecular Devices). Initial linkage-disequilibrium and quality-control assessments yielded a total of 19 informative single-nucleotide polymorphisms for genotyping and analysis in the full NIMH sample (7 single-nucleotide polymorphisms each in *ABPA1* and *ABCA1* and 5 single-nucleotide polymorphisms in *UBQLN1*). Four of the single-nucleotide polymorphisms in *UBQLN1* (rs2780995, UBQ-8i, rs2781002, and UBQ-10e) were also genotyped in the CAG families, and UBQ-8i was genotyped in the brain specimens for the functional analyses. Figure 2 shows the loca-



tion of *UBQLN1* variants, and further details are provided in the Supplementary Appendix.

**FUNCTIONAL ANALYSES OF UBQ-81 IN BRAIN SPECIMENS**

*RNA Extraction*

A portion of gray matter from the superior temporal sulcus (30 to 50 mg) was dissected from each brain under stringent RNase-free conditions according to previously published methods.<sup>12</sup> From each sample, RNA was extracted with Trizol reagent according to the manufacturer's instructions (Invitrogen) (see the Supplementary Appendix).

*Reverse-Transcriptase PCR*

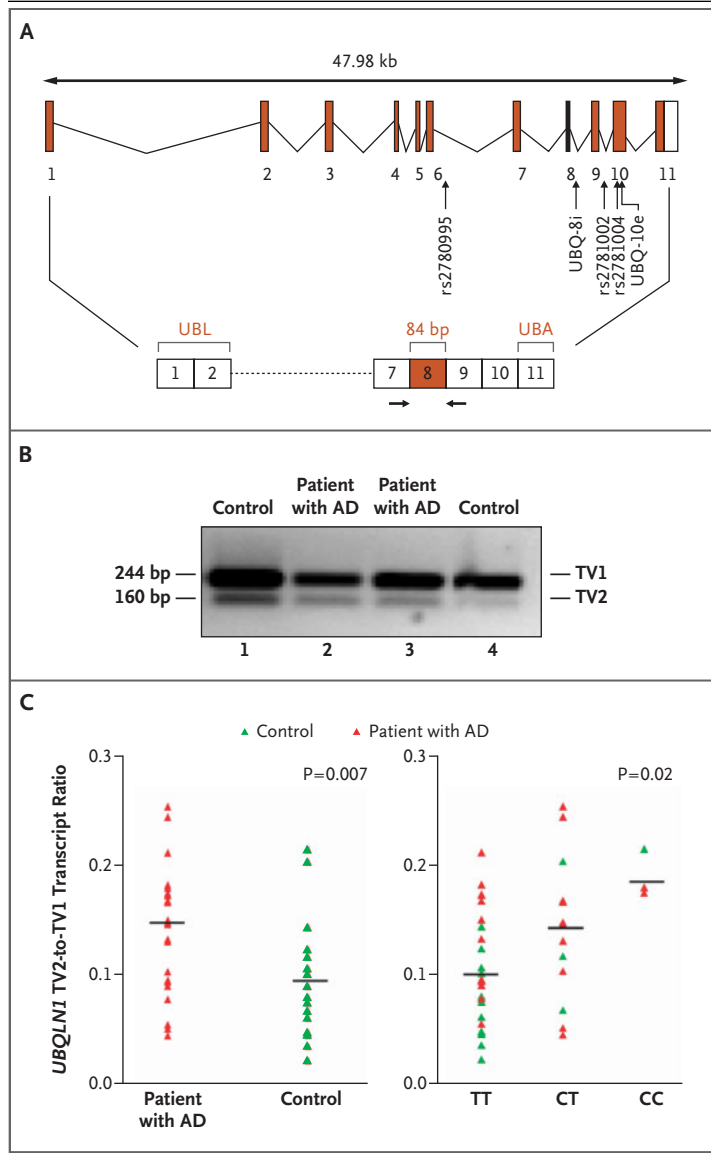
DNase-treated RNA (2 μg) was reverse transcribed to complementary DNA (cDNA) with the use of random hexamers according to the manufacturer's instructions (Invitrogen), but with 200 U of Superscript II reverse transcriptase (Invitrogen) used in each reaction.

*Quantitative Agarose-Gel Analysis of Transcript Variants 1 and 2 of UBQLN1*

*UBQLN1*-specific PCR primers from exon 7 and exon 9 were designed to detect transcript variant 1 (TV1; GenBank accession number, NM\_013438)

**Figure 2. Alternative Transcription of *UBQLN1* in Human Temporal Lobe.**

Panel A shows the exon and intron structure of *UBQLN1* transcript variant 1 (TV1), including all genotyped *UBQLN1* single-nucleotide polymorphisms (rs2780995 is located 1473 bp from the 3' end of exon 6, UBQ-8i is 70 bp from the 3' end of exon 8, rs2781002 is 67 bp from the 5' end of exon 9, rs2781004 is 1853 bp from the start codon in exon 10 [at codon 498 of TV1], and UBQ-10e is 1949 bp from the start codon in exon 10 [at codon 530 of TV1]). TV1 comprises 11 exons, whereas in transcript variant 2 (TV2), exon 8 is alternatively spliced out, without any resulting frameshift. *UBQLN1* has two structural motifs, a ubiquitin-like (UBL) domain and a ubiquitin-associated (UBA) domain, which are located at the N-terminal and C-terminal ends of the protein, respectively. These domains have been implicated in targeting and degeneration of proteins by the ubiquitin-proteasome pathway. The UBA domain is also part of the C-terminal amino acid sequence responsible for the binding of prenilins. Arrows indicate the locations of polymerase-chain-reaction (PCR) primers used to amplify TV1 and TV2. Panel B shows agarose-gel detection of TV1 and TV2 PCR fragments from human brain complementary DNA with the use of primers specific for exon 7 and exon 9. Amplification of TV1 and TV2 yielded 244-bp and 160-bp fragments, respectively. Sequence specificity of the PCR fragments for *UBQLN1* was confirmed by sequencing. AD denotes Alzheimer's disease. Panel C compares the ratio of TV2 to TV1 transcripts in temporal cortex from 25 patients with Alzheimer's disease and 17 control subjects (left), as well as with respect to UBQ-8i genotypes (right). The Mann-Whitney U test was used to compare the ratios of TV2 to TV1 transcripts between patients with Alzheimer's disease and control subjects; a one-way analysis of variance was used to compare the TV2-to-TV1 ratios among the UBQ-8i genotype groups. Twenty-six subjects had the TT genotype (13 of whom had Alzheimer's disease), 13 had the CT genotype (10 of whom had Alzheimer's disease), and 3 had the CC genotype (2 of whom had Alzheimer's disease), where C is the risk allele and T the alternative allele. Some of the symbols in the graphs overlap.



and transcript variant 2 (TV2; GenBank accession number, NM\_053067), which encompass and lack exon 8, respectively (the Supplementary Appendix lists PCR conditions and primer sequences). TV1 and TV2 PCR products were extracted from agarose gel and subjected to cycle sequencing to confirm their sequence specificity for *UBQLN1*. PCR reactions for complementary DNA from brain specimens from 25 patients with Alzheimer's disease and 17 controls (3 ng per microliter) were repeated four times, and mean values of the ratio of TV2 transcripts to TV1 transcripts were used for statistical analyses with respect to disease status and genotype data.

**STATISTICAL ANALYSIS**

Multipoint nonparametric linkage analyses were performed as described previously. In brief, we used GeneHunter-Plus and the ASM program (exponential model) to calculate Zlr scores and multipoint lod scores<sup>13,14</sup> (see the Supplementary Appendix).

Single-locus and multiloci association analyses were performed with the use of Family-Based Association Test (FBAT) software (version 1.5.3).<sup>15</sup> FBAT uses a generalized score statistic to perform a variety of tests similar to the transmission-disequilibrium test, including haplotype analyses, and the best results are obtained with the use of an ad-

divitive disease model, which was used here.<sup>15,16</sup> Haplotype analyses were performed on the basis of haplotype block predictions with the use of tagging single-nucleotide polymorphisms (described below). For all single-locus and haplotype analyses in FBAT, we used the empirical variance function of the program to account for the presence of linkage in the area<sup>17</sup> and an equal-weight offset correction to incorporate genotypes from both affected and unaffected subjects (see the FBAT Web site [at [www.biostat.harvard.edu/~fbat](http://www.biostat.harvard.edu/~fbat)] for details). All analyses included the full NIMH sample, the full CAG sample, and where available, both samples combined. All single-locus analyses were repeated with the use of an alternative family-based association program, the Pedigree disequilibrium test<sup>18,19</sup> (see the Supplementary Appendix).

#### *Linkage Disequilibrium and Haplotype Block Estimations*

Haplotype block predictions were based on the most complete genotype data available for the NIMH families (the full sample in the case of *APBA1* or *UBQLN1* and the screening sample in the case of *ABCA1*) with the use of the software programs HAP (which uses a dynamic programming algorithm based on imperfect phylogeny)<sup>20</sup> and Haploview (which is based on  $D'$ , the standardized disequilibrium parameter) (details are provided in the Supplementary Appendix).

#### *Selection of Families Showing Association with APBA1 and UBQLN1*

To assess the extent to which the implicated single-nucleotide polymorphisms in *APBA1* (rs1411438) and *UBQLN1* (UBQ-8i) contributed to the linkage signal on chromosome 9 with the extended set of markers, we used the "viewstat" option in FBAT to identify families in the full NIMH sample in which at least two risk alleles had been transmitted to subjects with Alzheimer's disease. This procedure specifically identifies families with data that are informative for the candidate single-nucleotide polymorphism and make the largest contribution to the association statistic (see the Supplementary Appendix). To test whether the association observed with UBQ-8i statistically accounted for the linkage signal on 9q22, we used the recently developed Genotype IBD (Identity-by-Descent) Sharing Test,<sup>21</sup> based on an additive disease model. Under the conditions we encountered (i.e., a disease allele frequency of 0.2, microsatellite-marker spacing of ap-

proximately 10 cM, and the inclusion of only 437 instead of 500 families), the statistical power of this test is moderate (i.e., 60 to 70 percent).

#### *Conditional Logistic Regression*

To provide an estimate of the magnitude of any potential effect on the risk of disease associated with the variants in *UBQLN1*, along with a rough indicator of the stability of this estimate, we performed conditional logistic regression stratified according to family<sup>22</sup> (see the Supplementary Appendix).

#### *Analysis of Messenger RNA Expression Data*

To assess differences in the ratio of TV2 transcripts to TV1 transcripts between patients with Alzheimer's disease and controls, we used the Mann-Whitney U test. To assess differences in the ratio of TV2 transcripts to TV1 transcripts among the different UBQ-8i genotypes, we used a one-way analysis of variance.

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## RESULTS

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Adding markers D9S1800 and D9S280 to the full genome screen allowed us to confirm and slightly refine our previous linkage results for chromosome 9q22 from a previous multipoint lod score of 2.75 at 99 cM to a multipoint lod score of 2.70 at 97 cM (Fig. 1A). The initial assessment of 58 single-nucleotide polymorphisms in the three candidate genes for Alzheimer's disease within the linked region yielded 19 informative variants that were genotyped and analyzed in the NIMH families (7 single-nucleotide polymorphisms each in *APBA1* and *ABCA1* and 5 single-nucleotide polymorphisms in *UBQLN1*). Single-locus analyses conducted with the use of FBAT revealed consistent and significant evidence of an association of Alzheimer's disease with two single-nucleotide polymorphisms in *UBQLN1* (UBQ-8i [ $P=0.028$ ] and rs2781002 [ $P=0.046$ ]) (Table 1), one single-nucleotide polymorphism in *APBA1* (rs1411438 [ $P=0.04$ ]) (Table 1 in the Supplementary Appendix), but no signal in *ABCA1* (Table 2 in the Supplementary Appendix). These findings were also detected with the use of the pedigree disequilibrium test (Table 1).

To delineate the ancestral haplotype architecture giving rise to these putative disease variants, we estimated the underlying haplotype block structure using HAP and Haploview. These analyses consistently showed that a single haplotype block encompassed all single-nucleotide polymorphisms

**Table 1. Tests of Association and Conditional Logistic-Regression Analyses of Single-Nucleotide Polymorphisms in *UBQLN1* and Alzheimer's Disease.\***

Test (No. of Families)	rs2780995	UBQ-8i	rs2781002	rs2781004	UBQ-10e
<b>FBAT — z score (P value)†</b>					
NIMH group (n=437)	-1.1 (0.3)	2.2 (0.03)	2.0 (0.046)	0.2 (0.83)	-0.9 (0.4)
CAG group (n=217)	-1.9 (0.05)	2.0 (0.047)	1.8 (0.07)	ND	0.4 (0.7)
Combined group (n=654)	-2.0 (0.04)	3.0 (0.003)	2.7 (0.008)	ND	0.5 (0.6)
<b>Pedigree disequilibrium test — chi-square (P value)‡</b>					
NIMH group (n=437)	1.8 (0.21)	5.0 (0.03)	2.9 (0.09)	0.5 (0.50)	0.4 (0.50)
CAG group (n=217)	2.9 (0.09)	3.5 (0.06)	2.9 (0.09)	ND	0.1 (0.76)
Combined group (n=654)	4.7 (0.03)	8.3 (0.004)	5.7 (0.02)	ND	0.1 (0.82)
<b>Conditional logistic regression — odds ratio (95% CI)§</b>					
NIMH group					
2-Allele homozygotes vs. 1-allele homozygotes	1.3 (0.7–2.3)	2.3 (1.0–5.4)	2.1 (1.1–3.9)	0.8 (0.3–2.6)	1.3 (0.6–2.8)
2-Allele heterozygotes vs. 1-allele homozygotes	1.0 (0.6–1.8)	1.4 (1.0–2.1)	0.9 (0.7–1.4)	0.7 (0.2–2.0)	1.1 (0.5–2.2)
CAG group					
2-Allele homozygotes vs. 1-allele homozygotes	1.8 (0.8–4.4)	2.1 (0.7–6.9)	1.7 (0.7–3.9)	ND	1.5 (0.5–4.9)
2-Allele heterozygotes vs. 1-allele homozygotes	1.5 (0.7–4.0)	1.9 (1.1–3.5)	1.6 (0.9–2.8)	ND	2.5 (0.8–7.1)
Combined group					
2-Allele homozygotes vs. 1-allele homozygotes	1.4 (0.9–2.2)	2.1 (1.1–4.0)	1.8 (1.1–3.0)	ND	1.4 (0.7–2.5)
2-Allele heterozygotes vs. 1-allele homozygotes	1.1 (0.7–1.7)	1.5 (1.1–2.0)	1.1 (0.8–1.5)	ND	1.4 (0.8–2.4)

\* FBAT denotes Family-Based Association Test, NIMH National Institute of Mental Health, CAG Consortium on Alzheimer's Genetics, ND no data, and CI confidence interval.

† Positive z scores for minor alleles indicate an increased rate of transmission among subjects with Alzheimer's disease. Minor-allele frequencies in the NIMH sample and the CAG sample were 0.38 and 0.34, respectively, for rs2780995 (C); 0.22 and 0.26, respectively, for UBQ-8i (C); 0.37 and 0.44, respectively, for rs2781002 (G); 0.15 and not available, respectively, for rs2781004 (A); and 0.26 and 0.26, respectively, for UBQ-10e (T). The z scores for the risk-associated haplotype H3 were 2.0 (P=0.048) for the NIMH sample, 1.8 (P=0.07) for the CAG sample, and 2.7 (P=0.006) for the combined sample. For details on haplotype configurations and frequencies, see Table 3 in the Supplementary Appendix. The empirical variance option in FBAT was used to account for the presence of linkage (see the Methods section in the Supplementary Appendix).

‡ The global average statistic was used in the pedigree disequilibrium test. P values are based on 1 degree of freedom.

§ Conditional logistic regression (stratified according to family) was adjusted for the presence or absence of *APOE* ε4 (NIMH sample only), sex, and age. 2-Allele indicates alleles that are overtransmitted to affected subjects (risk alleles). ND indicates that this single-nucleotide polymorphism was not genotyped in the CAG sample. The CIs may be slightly too narrow because this approach may underestimate the standard errors when multiple affected and unaffected subjects are included in each family.

in *UBQLN1*, with four major haplotypes (Fig. 1; details in Tables 3 and 7 in the Supplementary Appendix) that could be tagged by three single-nucleotide polymorphisms. Haplotype analyses with these single-nucleotide polymorphisms revealed a single haplotype (H3) with a significantly increased rate of transmission to affected subjects in the unstratified NIMH sample (P=0.048). This risk haplotype was almost exclusively defined by the risk allele of UBQ-8i (C allele). The haplotype structure within *APBA1* appeared more complex (Fig. 1), with only one haplotype block in the 3' half of the gene predicted by both algorithms. Testing three haplotype-tagging single-nucleotide polymorphisms within this block did not reveal evidence of an asso-

ciation with Alzheimer's disease (Table 1 in the Supplementary Appendix).

Next, we addressed the extent to which the observed associations in *APBA1* (rs1411438) and *UBQLN1* (UBQ-8i) accounted for the linkage signals on chromosome 9. For this purpose, we identified families in the NIMH sample in which at least two risk alleles for either rs1411438 or UBQ-8i had been transmitted to affected subjects and removed these pedigrees from the linkage analyses. For rs1411438, a total of 37 families (8 percent of the NIMH sample) were excluded, reducing the multipoint lod score only slightly, from 2.7 to 2.6 at 9q22 and from 1.3 to 1.2 at 9p21 (Fig. 1 in the Supplementary Appendix). In contrast, after exclud-

ing the 27 families with the strongest association with UBQ-8i (6 percent), we observed a substantial change in the linkage signal, with a decrease in the multipoint lod score from 2.7 to 1.6 at 9q22 and from 1.2 to 0.9 at 9p21 (Fig. 1A). Conversely, when the genome-screen markers were analyzed only in the 27 families with a UBQ-8i association, the multipoint lod score increased to 2.9 and was maximal near 89 cM (approximately 83 Mb) — less than 2 Mb from the predicted location of *UBQLN1* (Fig. 1A). These analyses were supported by the results of the Genotype IBD Sharing Test, which yielded a P value of 0.1 (nonparametric linkage score, 2.0) after accounting for the association with UBQ-8i, suggesting that a substantial portion of the 9q22 linkage signal is caused by the observed association within *UBQLN1*, but not with *APBA1*.

Consistent with our findings in the larger NIMH sample, we detected an increased rate of transmission of the H3 haplotype among affected subjects in the independent CAG sample ( $P=0.07$ ) (Table 1). More important, single-locus tests with FBAT revealed a significant association with the same alleles of UBQ-8i ( $P=0.047$ ) and rs2780995 ( $P=0.05$ ), as well as the suggestion of such an association with rs2781002 ( $P=0.07$ ); these findings were also detected with the use of the pedigree disequilibrium test (Table 1). In contrast to the positive findings obtained with *UBQLN1*, no evidence of an association was detected with rs1411438 in *APBA1* in the CAG families ( $P=0.94$ ) (Table 1 in the Supplementary Appendix).

Merging the two groups of families revealed that UBQ-8i, followed by rs2781002 and rs2780995, showed the most pronounced single-locus signals ( $P=0.003$ ,  $P=0.008$ , and  $P=0.04$ , respectively) (Fig. 1 and Table 1). The use of conditional logistic regression to estimate the size of the effect after adjustment for the presence or absence of *APOE*  $\epsilon 4$ , age, and sex revealed a significant and dose-dependent increase in the risk of Alzheimer's disease among carriers of one or two copies of the UBQ-8i risk allele, as compared with those with no copies of this allele (respective odds ratio, 1.5 [95 percent confidence interval, 1.1 to 2.0] and 2.1 [95 percent confidence interval, 1.1 to 4.0]) (Table 1), compatible with an additive mode of inheritance. No interaction was observed with *APOE*  $\epsilon 4$ , sex, or age at onset, although most positive signals tended to be more pronounced in families with late-onset Alzheimer's disease than in those with early-onset Alzheimer's disease (data not shown). Finally, since the

majority of families in both samples were white (see the Supplementary Appendix), the results changed only minimally when these families were analyzed separately.

The two single-nucleotide polymorphisms showing the strongest genetic effects (UBQ-8i and rs2781002) map near exon–intron boundaries (Fig. 2A). In particular, UBQ-8i is close to exon 8, 70 bp downstream of its 3' splice site. Exon 8 is absent in an alternative, in-frame transcript, here denoted as TV2. We therefore assessed whether the UBQ-8i risk allele affects the splicing of this exon in the *UBQLN1* message in brain. Specifically, we measured the relative abundance of the two transcript variants (TV2 and TV1) in RNA extracts from brain tissue of 25 patients with neuropathologically confirmed Alzheimer's disease and 17 age-matched controls with no evidence of Alzheimer's disease on postmortem examination. As can be seen in Figure 2B, both transcript variants were expressed in brain, with TV1 levels generally exceeding those of TV2 by a factor of approximately 10. To quantify both PCR products reliably, we determined the linear range of amplification for TV1 and TV2 by logarithmically relating the intensities of PCR fragments to the number of PCR cycles. On the basis of this analysis, the optimal number of PCR cycles was determined to be 37, which allowed us to quantify both amplicons without reaching the plateau phase of PCR.

Quantification of both variants from agarose gels revealed a significant increase in the ratio of TV2 to TV1 in patients with Alzheimer's disease as compared with controls ( $P=0.007$ ). Genotyping of this sample showed the UBQ-8i risk allele to be overrepresented among patients with Alzheimer's disease as compared with controls (28.0 percent vs. 14.7 percent). Although this difference did not reach statistical significance ( $P=0.15$  by the chi-square test) owing to the small sample size, it is consistent with the UBQ-8i risk effect observed in the NIMH and CAG families. In view of this result, we next determined whether the observed alteration in the transcript ratio was associated with the UBQ-8i risk allele independently of disease status. If so, one would expect to observe a risk-allele-dependent increase in the ratio of TV2 to TV1 both in patients with Alzheimer's disease and controls. As can be seen in Figure 2C, the increase in TV2 relative to that in TV1 was more pronounced among all carriers of the UBQ-8i risk allele, regardless of disease status. Moreover, the extent of splicing

was dependent on the number of risk alleles. As compared with the ratio of TV2 to TV1 splice variants in brain tissue from 26 subjects with no risk alleles, the ratio increased by an average factor of approximately 1.4 among 13 heterozygous carriers and a factor of approximately 1.8 among 3 homozygous carriers ( $P=0.02$ ) (Fig. 2C). Collectively, these data revealed a dose-dependent relationship between the UBQ-8i risk allele and the increased ratio of TV2 to TV1 in brain tissue, regardless of Alzheimer's disease status.

#### DISCUSSION

Ubiquitin 1 was initially described as a "protein linking integrin-associated protein . . . with cytoskeleton."<sup>23</sup> It contains a ubiquitin-like domain at its N terminal and a ubiquitin-associated domain at its C terminal,<sup>24</sup> and thus it can interact with both the proteasome and ubiquitin ligases.<sup>25</sup> In this capacity, ubiquitin 1 has been shown to inhibit the *in vivo* degradation of several ubiquitin-dependent proteasome substrates, including p53, the  $\text{I}\kappa\text{B}\alpha$ ,<sup>25</sup> and the  $\alpha$  and  $\beta$  subunits of the  $\gamma$ -aminobutyric acid receptor.<sup>26</sup> Further evidence of a role for ubiquitin 1 in regulating protein degradation comes from a report that it interacts with the mammalian target of rapamycin protein kinase.<sup>27</sup>

More specific to Alzheimer's disease, ubiquitin 1 has also been shown to interact with presenilin 1 and presenilin 2 through its C-terminal ubiquitin-associated domain and to promote the accumulation of presenilin *in vitro*.<sup>24</sup> Consistent with this observation is the finding that cells overexpressing both ubiquitin 1 and presenilin 2 had a lower rate of turnover of high-molecular-weight forms of presenilin 2 owing to its decreased ubiquitination.<sup>28</sup> Another study has suggested a protective role for ubiquitin in the regulation of hypoxia-induced death of astroglial cells through the suppression of c/EBP homologous protein, possibly requiring the interaction of ubiquitin 1 with protein-disulfide isomerase.<sup>29</sup> Although it remains unclear whether an interaction between ubiquitin and protein-disulfide isomerase is required to protect against apoptosis induced by c/EBP homologous protein, exon 8 of *UBQLN1* encodes part of the putative domain needed to interact with protein-disulfide isomerase. Thus, the transcript lacking exon 8, *UBQLN1* TV2, which was increased in the brains of those carrying the UBQ-8i allele, is unlikely to bind protein-disulfide isomerase.

All the *P* values we reported for the genetic analyses are nominal (i.e., not corrected for multiple comparisons). However, several points suggest that our results reflect a genuine association between *UBQLN1* and Alzheimer's disease. First, we found a positive association between Alzheimer's disease and multiple adjacent single-nucleotide polymorphisms in *UBQLN1*. Second, we showed that the observed association with UBQ-8i, but not with rs1411438 in *APBA1*, accounts for a substantial portion of the linkage signal in this region of chromosome 9 that we and other groups have observed. Finally, and most important, we demonstrated consistent risk effects with the same single-locus and haplotype alleles in two independent cohorts of families with Alzheimer's disease and found significantly increased levels of an alternatively spliced *UBQLN1* transcript in the brains of carriers of the UBQ-8i risk allele.

Notwithstanding the significant and consistent effects we observed throughout the various experiments, further replication in independent family-based and case-control samples is required to confirm the role of *UBQLN1* as a bona fide genetic risk factor for Alzheimer's disease. However, it is unlikely that this can be achieved with the sample sizes typically used to evaluate the well-established association between *APOE*  $\epsilon 4$  and Alzheimer's disease — 200 subjects, or even fewer.<sup>30</sup> A meta-analysis of the effect of the *APOE*  $\epsilon 4$  allele in whites revealed odds ratios of 3 and 15 in heterozygous and homozygous carriers, respectively.<sup>30</sup> In our full-genome screen, these associations translated into a significant multipoint lod score of 4.2 on chromosome 19q13 in the unstratified NIMH sample, near the location of *APOE*.<sup>5</sup> In contrast, the odds ratios related to the UBQ-8i risk effect found in this study were lower and ranged from 1.4 to 2.3, giving rise to a multipoint lod score of 2.8 in the corresponding chromosomal region. Thus, the number of patients and controls that will probably be required to detect the *UBQLN1* association reported here would need to exceed those used to detect the *APOE* effects by severalfold. Along these lines, a recent study assessing the statistical power to detect moderate genetic effects estimated that — even under favorable circumstances — at least 550 patients and 550 controls would be needed to detect an effect size similar to the one observed here for UBQ-8i.<sup>31</sup> In the Alzheimer's disease field, the majority of studies conducted in an effort to confirm disease associations typically have samples that are

less than half this size. This may explain the rampant inconsistencies encountered in genetic analyses of putative candidate genes for Alzheimer's disease in the literature to date.<sup>6</sup> Additional genetic studies involving both family-based and large case-control samples, along with more extensive functional testing of *UBQLN1*, will be required to characterize the potential effect of this gene on the risk of Alzheimer's disease.

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Dr. Bertram reports being a consultant to Neurogenetics, and hold-

ing equity or stock options with Neurogenetics and Prana Biotechnology. Ms. Elliott, Dr. Becker, Dr. Velicebi, and Dr. Wagner report being employees of Neurogenetics and holding stock options in Neurogenetics. Ms. Elliott, Dr. Becker, and Dr. Wagner report having assigned to Neurogenetics their inventions made as employees of the company and having received no royalties from the company for these inventions. Ms. Elliott, Dr. Velicebi, and Dr. Wagner also report holding stock in Merck. Dr. Hyman reports being a consultant or serving on the scientific advisory boards of Elan, Forest Laboratories, Takeda Pharmaceuticals, FoldRx Pharmaceuticals, and Neuro Diagnostics Consulting and having received lecture fees from Elan. Dr. Tanzi reports being a consultant or serving on the scientific advisory board and board of directors of Neurogenetics and Prana Biotechnology; holding equity or stock options with Neurogenetics, Prana Biotechnology, and Elan; and having received consulting or lecture fees from Novartis, Aventis Pharma, Eisai, and PureTech Ventures.

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