R47H Variant of TREM2 Associated With Alzheimer Disease in a Large Late-Onset Family Clinical, Genetic, and Neuropathological Study

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IMPORTANCE The R47H variant in the triggering receptor expressed on myeloid cells 2 gene (TREM2), a modulator of the immune response of microglia, is a strong genetic risk factor for Alzheimer disease (AD) and possibly other neurodegenerative disorders.

OBJECTIVE To investigate a large family with late-onset AD (LOAD), in which R47H cosegregated with 75% of cases.

DESIGN, SETTING, AND PARTICIPANTS This study includes genetic and pathologic studies of families with LOAD from 1985 to 2014. A total of 131 families with LOAD (751 individuals) were included from the University of Washington Alzheimer Disease Research Center. To identify LOAD genes/risk factors in the LOAD123 family with 21 affected members and 12 autopsies, we sequenced 4 exomes. Candidate variants were tested for cosegregation with the disease. TREM2 R47H was genotyped in an additional 130 families with LOAD. We performed clinical and neuropathological assessments of patients with and without R47H and evaluated the variant’s effect on brain pathology, cellular morphology, and expression of microglial markers.

MAIN OUTCOMES AND MEASURES We assessed the effect of TREM2 genotype on age at onset and disease duration. We compared Braak and Consortium to Establish a Registry for Alzheimer’s Disease scores, presence of α-synuclein and TAR DNA-binding protein 43 aggregates, and additional vascular or Parkinson pathology in TREM2 R47H carriers vs noncarriers. Microglial activation was assessed by quantitative immunohistochemistry and morphometry.

RESULTS Twelve of 16 patients with AD in the LOAD123 family carried R47H. Eleven patients with dementia had apolipoprotein E E4 (ApoE4) and R47H genotypes. We also found a rare missense variant, D353N, in a nominated AD risk gene, unc-5 homolog C (UNC5C), in 5 affected individuals in the LOAD123 family. R47H carriers demonstrated a shortened disease duration (mean [SD], 6.7 [2.8] vs 11.1 [6.6] years; 2-tailed t test; P = .04) and more frequent α-synucleinopathy. The panmicroglial marker ionized calcium-binding adapter molecule 1 was decreased in all AD cases and the decrease was most pronounced in R47H carriers (mean [SD], in the hilus: 0.114 [0.13] for R47H_AD vs 0.574 [0.26] for control individuals; 2-tailed t test; P = .005 and vs 0.465 [0.32] for AD; P = .02; in frontal cortex gray matter: 0.006 [0.004] for R47H_AD vs 0.016 [0.01] for AD; P = .04 and vs 0.033 [0.013] for control individuals; P < .001). Major histocompatibility complex class II, a marker of microglial activation, was increased in all patients with AD (AD: 2.5, R47H_AD: 2.7, and control: 1.0; P < .01).

CONCLUSIONS AND RELEVANCE Our results demonstrate a complex genetic landscape of LOAD, even in a single pedigree with an apparent autosomal dominant pattern of inheritance. ApoE4, TREM2 R47H, and rare variants in other genes, such as UNC5C D353N, are likely responsible for the notable occurrence of AD in this family. Our findings support the role of the TREM2 receptor in microglial clearance of aggregation-prone proteins that is compromised in R47H carriers and may accelerate the course of disease.

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Alzheimer disease (AD) is the most common form of age-related dementia. Typically, AD manifests after 65 years of age (late-onset AD [LOAD]), although 5% to 10% of patients have early-onset AD. Approximately 13% of early-onset AD cases are mendelian dominant forms of the disease caused by high-penetrance variants in amyloid protein and presenilins. Although LOAD is traditionally referred to as sporadic, familial clustering has frequently been observed and biological relatives of patients with LOAD are at an increased risk for developing dementia. A twin study estimated the heritability of AD to be as high as 79%. Whole-genome association studies have identified multiple genomic loci/genes that appear to influence disease onset/progression; however, only 2 of these genetic factors are associated with a significant disease risk: the ε4 allele of apolipoprotein E (ApoE4) (odds ratio, 3.2) and the R47H variant in the triggering receptor expressed on myeloid cells 2 gene (TREM2) (odds ratios, 1.7-4.5). Whereas ApoE4 is common in the white population (minor allele frequency, 12.6%), TREM2 R47H is rare (minor allele frequency, 0.26%; http://evs.gs.washington.edu/EVS/). In the brain, TREM2 is expressed by microglia. To our knowledge, segregation of R47H carriers and demonstrate mutation-specific changes in neuropathology and expression of microglial markers in brains.

Methods

Participants
Families with LOAD and control individuals were ascertained and evaluated through the University of Washington Alzheimer Disease Research Center. Examinations, blood sampling, medical record reviews, brain autopsies, and genetic analyses were performed under protocols approved by the institutional review boards of the University of Washington and the Seattle Veterans Affairs Puget Sound Health Care System. Written informed consent was obtained from participants.

Exome Sequencing, Copy-Number Variation Analysis, and Genotyping
Target enrichment was performed by hybridization of shotgun fragment libraries to custom microarrays or to NimbleGen solution_V2refseq2010.HG19 probe library. Exome sequencing was performed as described previously. Sanger sequencing was used to confirm rare exome variants or evaluate them in other family members. For copy-number variant detection, a customized microarray (Agilent 2 × 400K probes) was designed to detect copy-number variants greater than 10 kbp within 1367 sites predisposed for genomic rearrangements and greater than 50 kbp over the rest of the genome. Array comparative genomic hybridization and analysis were performed as described previously and compared with data from 8900 control individuals. R47H was genotyped using a TaqMan single-nucleotide polymorphism assay (rs7592628 Assay-by-Design; Applied Biosystems/Life Technologies).

Postmortem Brain Tissues
Autopsy tissues from members of the LOAD123 family, sporadic LOAD cases, and age-matched nondemented (ND) control individuals were obtained from the University of Washington Neuropathology Core Brain Bank. The average age of the individuals was 84.9 years and the average postmortem interval was 4.5 hours.

Immunohistochemistry
Formaldehyde-fixed paraffin-embedded sections were deparaffinized and autoclaved at 15 psi and 121°C for 20 minutes in citrate buffer pH of 6.0 for antigen retrieval. Immunodetection was performed with antibodies against α-synuclein (LB509, from John Trojanowski, MD, PhD, University of Pennsylvania), TAR DNA-binding protein 43 (TDP-43) (10782-2-AP; ProteinTech), major histocompatibility complex II (MHCII) (M0746; Dako/Agilent Technologies), and ionized calcium-binding adapter molecule 1 (Iba1) (019-19741; Wako), and the correspondent secondary antibodies (Vector Laboratories). The specificity of antigen detection was ascertained by omitting the primary antibody. α-Synuclein and TDP-43 deposits were scored qualitatively per each brain region (0 = absence; 1 = presence). For each group, the severity of deposition was calculated as a sum of qualitative scores per region and normalized by the number of samples. The deposit frequency was determined as the number of patients with deposition in at least 1 region. P values were calculated using the Fisher exact test.

Imaging and Quantitative Analysis of Diamino Benzidine-Stained Tissue Sections
Immunolabeled sections were analyzed using MicroComputer Imaging Device (Imaging Research). Blinded assessment of optical density measurements were obtained relative to the proportional area for Iba1 and MHCII immunostaining. Immunoreactivity within the hippocampal formation was assessed in the hilus, cornu ammonis region 1, parahippocampal gyrus, and white matter (average of 3 separate readings per region). All data are represented as mean ± SEM. A 2-tailed t test was used to assess differences. For graded immunohistochemistry analysis, blinded assessment was performed by 2 observers. Five fields in hippocampal neuropil were counted per individual. The relative intensity and pattern of immunostaining in neuropil were graded on a scale of 0 to 3 for both Iba1 and MHCII, incorporating quantitative evaluation of immunopositive cells and observable phenotype. Major histocompatibility complex class II labeling graded zero had no immunoreactivity; grade 1 signified observable, although few, immunopositive cells per field; grade 2 represented multiple immunopositive cells per field, with fewer than half demonstrating an activated plump morphology, with thicker processes and more bizarre shapes; and grade 3 was given where a field was prominently immunopositive for numerous MHCII cells, many appearing activated. Staining with Iba1 was ranked on presence in white matter and morphology. Completely absent Iba1 labeling received a zero grade. Grade 1 fields showed virtually no Iba1-positive staining, grade 2 fields contained a few Iba1-positive cells in white matter in addition to
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Results

TREM2 R47H in Familial LOAD

The LOAD123 family contains 21 individuals with an AD diagnosis (Figure 1; eTables 1 and 2 in the Supplement). The mean (SD) age at onset was 72.5 (8.2) years, the mean (SD) age at death was 81 (8.4) years, and the mean (SD) disease duration was 7.7 (4.4) years. The pedigree in Figure 1 shows vertical disease transmission to the 3 branches. There is age-at-onset heterogeneity, clustered at early age (60-70 years) but extending to 88 years.

We selected 4 affected individuals for exome sequencing (Figure 1). Two of them, III-5 and III-12, were additionally screened for structural genome variation, but no shared pathogenic uncommon structural variants were identified (eTable 3 in the Supplement). Shared exome variants were sorted by frequency in the population assuming that LOAD risk variants of strong effect would be uncommon (<5%) or rare (<1%). Five shared identity-by-descent regions contained 9 missense variants with frequency less than 5% (eTable 4 in the Supplement). None of the exomes had rare missense variants in the genes known to cause early-onset AD or frontotemporal dementia.18 One exome contained a rare missense variant, D353N, in the unc-5 homolog C gene (UNC5C) (rs145150541; minor allele frequency, 0.2% in white individuals; http://evs.gs.washington.edu/EVS/), in which another rare variant (rs137875858) was reported in association with AD.14 D353N is predicted to be damaging to the protein (PolyPhen2 score, 0.85).

The R47H variant in TREM2 was prioritized based on association studies.6,7 Sanger sequencing confirmed R47H presence in 11 of 15 affected individuals with available DNA and case II-3 could be inferred as a carrier based on the pedigree structure, for a total of 12 R47H carriers of 16 patients with AD (75%). In the central branch of the family, of 9 siblings with AD, 5 inherited this variant, and 3 of these transmitted it to their affected offspring. Three unaffected individuals were also deduced or shown to carry the variant: II-4 and III-14, who died before the typical age of LOAD onset (57 and 62 years), and III-20, who died at age 87 (Figure 1). Four other siblings with AD were negative for R47H and did not transmit the disease to their 12 offspring (age range, 64-86 years).

We studied the distribution of ApoE and UNC5C alleles in the LOAD123 family. Fourteen of 15 affected individuals and 4 of 10 unaffected individuals had at least 1 ApoE4 allele. Of the 3 individuals who were APOE 4/4 homozygotes, 2 with AD had quite disparate ages at onset—60 and 82 years (IV-55 and III-17)—and the third was unaffected at 74 years (IV-57). UNC5C D353N was transmitted within the central branch of the family; of 8 siblings with AD with available DNA, 3 inherited this variant (III-11, III-13, and III-18) and 1 (III-11) transmitted it to her affected offspring (IV-26 and IV-29).

We genotyped R47H in an additional 130 families with LOAD (713 individuals). Eight R47H carriers were found in 3...
white European families (KS, MGK, and #62468) and 1 Japanese family (NOA). Seven of 8 R47H carriers had an AD diagnosis. Of the individuals with AD, 1 of 1 in KS, 2 of 4 in NOA, 2 of 3 in MGK, and 2 of 5 in #62468 had R47H. Assuming 1 founder per family, R47H was present in 3% of familial LOAD cases, 10 times higher than in the general population (http://evs.gs.washington.edu/EVS/).

In the 5 relevant families, we assessed the effect of TREM2 genotype on age at onset and duration of disease. The disease onset did not differ between R47H carriers and noncarriers (mean [SD], 73.3 [7.6] years; range, 58–85 years; n = 16 and 73.8 [9.7] years; range, 58–90 years; n = 18, respectively). As expected, disease onset was significantly earlier in the patients with at least 1 ApoE4 allele (mean [SD], 70.74 [7.9] years; range, 56–85 years; n = 23 vs 80.9 [5.8] years; range, 71–90 years; n = 10 for noncarriers of the ApoE4 allele; 2-tailed t test P = .0004). The ApoE genotype of 1 affected patient was not tested and could not be deduced. In the subset of patients with AD with at least 1 ApoE4 allele, the TREM2 genotype did not further reduce the age at onset (mean [SD], 72.9 [6.2] years; range, 60–85 years; n = 13 vs 67.9 [5.3] years; range, 58–82 years; n = 10 for R47H carriers and noncarriers, respectively).

Disease duration did not differ between ApoE4 carriers and noncarriers (mean [SD], 9.4 [6.2] years; range, 3–23 years; n = 20 and 7.5 [3.1] years; range, 3–10 years; n = 4, respectively). However, disease duration was significantly shorter in the TREM2 R47H AD group as a whole (mean [SD], 6.7 [2.8] years; range, 3–11 years; n = 11 vs 11.1 [6.6] years; range, 3–23 years; n = 14 for noncarriers; 2-tailed t test P = .04). This disease duration effect was also seen in the subgroup of ApoE4 carriers with the R47H variant (mean [SD], 6.3 [2.6] years; range, 3–11 years; n = 10) vs those positive for ApoE4 without the R47H variant (mean [SD], 12.5 [7.2] years; range, 5–23 years; n = 10; 2-tailed t test P = .03). The small number of patients with AD with ApoE 3/3 genotype and TREM2 R47H (n = 2) precluded a statistical test of age-onset effects of R47H, but there was no apparent change. These 2 patients had onset at 78 and 81 years (mean, 79.5 years) compared with a range of 71 to 90 years (n = 8; mean [SD], 81.3 [6.5] years) for patients with ApoE 3/3 and without TREM2 R47H. It was not possible to assess the effect of R47H on disease duration in the absence of an ApoE4 allele as both patients are still living.

### Clinical and Neuropathological Findings in the LOAD123 Family

Clinical presentations of the patients with AD positive and negative for R47H were similar; most patients showed slowly progressive dementia with memory problems as the initial symptom. Initial psychosocial signs were present in 2 patients: a TREM2 R47H carrier (III-15) had hallucinations and aggression early in the disease and a noncarrier (III-9) developed severe paranoia. Another R47H carrier (III-11) was diagnosed as having Parkinson disease (PD) 3 years after the onset of the memory deficit.

Autopsies were performed on 12 patients, 10 with clinically diagnosed dementia and 2 unaffected relatives (Table). Neuropathologic examination confirmed the diagnosis of AD in 9 patients with dementia with Braak stages of 5 or 6 (9 of 10) and Consortium to Establish a Registry for Alzheimer’s Disease...
ders including frontotemporal dementia, PD, and amyotrophic lateral sclerosis were mostly common in the substantia nigra and the amygdala (6 of 8 with AD; 1 of 2 ND) and less common in the substantia nigra (4 of 8 with AD; 1 of 2 ND), medulla (3 of 8 with AD; 1 of 2 ND), and frontal cortex (3 of 8 with AD; 0 of 2 ND). Both III-11 and III-15 had Lewy bodies in the substantia nigra, mild neuronal loss, and gliosis suggestive of PD-like pathology. Scoring of α-synuclein deposition in the 5 brain regions indicated that α-synuclein inclusions were more frequent (P = .06) and more severe (P = .02) in R47H carriers compared with their noncarrier relatives. Inclusions of TDP-43 were observed in 5 of the LOAD123 AD cases, 4 of these with neocortical pathologic changes. Both ND relatives also had TDP-43 deposits in the amygdala, and the one without the R47H variant had additional pathology in the parahippocampal gyrus and the frontal cortex. Scoring of TDP-43 deposits in 4 regions did not reveal differences between R47H carriers and noncarriers.

Two women were of particular interest. The person with earliest age at onset (IV-55, 60 years) carried both APOE 4/4 and TREM2 R47H. A female relative (III-17) with the same genotype had much later onset at 82 years. Both patients had a Braak stage of 5 and a CERAD score of C and neither had vascular pathology.

Decreased Iba1 Level Is a Characteristic Feature of TREM2 R47H Microglia

To analyze the cellularity and activation state of microglia, we performed immunohistochemistry of postmortem brains with panmicroglial marker Iba1 and activation-specific marker MHCII. We chose the hippocampus as a representative area affected in AD and as a region with the highest number of microglia cells. Three groups were compared: (1) patients with AD heterozygous for R47H (R47H_AD group); (2) patients with AD with normal TREM2 genotype (AD group); and (3) age-matched ND control individuals (ND group) (eTable 5 in the Supplement). The Iba1 signal was reduced in all AD cases (Figure 2A). In the hilus, Iba1 reactivity in the R47H_AD group differed from both control individuals (mean [SD], 0.574 [0.26] for control individuals vs 0.114 [0.13] for R47H_AD; 2-tailed t test; P = .005) and AD patients with normal TREM2 (mean [SD], 0.465 [0.32] for AD vs 0.114 [0.13] for R47H_AD; 2-tailed t test; P = .02). In white matter, the Iba1 level of both AD groups was significantly decreased (mean [SD], 1.345 [0.28] for control individuals vs 0.247 [0.27] for R47H_AD; 2-tailed t test; P < .001 and 1.345 [0.28] for control individuals vs 0.522 [0.25] for AD; 2-tailed t test; P = .003). In contrast, the MHCII level was substantially higher in both AD groups compared with ND control individuals (Figure 2B).

We additionally performed Iba immunostaining of frontal lobe sections and confirmed the reduction of the Iba1 level in the R47H_AD cases compared with the AD and ND groups that reached significance in gray matter (mean [SD], 0.016 [0.01] for AD vs 0.006 [0.004] for R47H_AD; 2-tailed t test; P = .04 and 0.033 [0.013] for control individuals vs 0.006 [0.004] for R47H_AD; 2-tailed t test; P < .001) (Figure 3). In addition to per-area analysis, we performed per-large-object quantification (where large objects corresponded to cell bodies; see the eAppendix in the Supplement). There was a strong correlation between the total positive area and the total positive area of large (cell-body sized) particles. In R47H_AD cases with decreased density of Iba1 signal per
Although it has been established by population-based studies that the R47H variant of TREM2 is a risk factor for AD, to our knowledge, the effect of this variant at the family level has not been described. We have characterized a large multigen-
pathogenic TREM2 variants found in a spectrum of neurodegenerative disorders,\textsuperscript{18-21,25-29} indicating an importance of this receptor for neuronal health. Our study further supports its role as a risk factor in familial LOAD. Consistent with some,\textsuperscript{6} but not all, studies,\textsuperscript{6,19} we did not observe an R47H influence on age at onset. However, we noticed that our patients with AD with R47H had shortened disease duration that presumably reflects an accelerated disease progression. The link between R47H and the ultimate life expectancy of patients with AD awaits confirmation in a larger sample.

Ionized calcium-binding adapter molecule 1 has been considered a proinflammatory protein expressed by both resting and activated microglial cells. It is used as a robust marker to characterize microglia. Ionized calcium-binding adapter molecule 1 is highly upregulated in microglia in murine models of acute neuroinflammation\textsuperscript{29,30} and in a mouse microglial cell line on stimulation with β-amyloid 42 peptide.\textsuperscript{31} In contrast to acute neuroinflammation, we demonstrated here that the chronic activated phenotype adopted by AD microglia is characterized by a substantial decrease of Iba1 level, and this lowered expression is exacerbated in R47H carriers. Our observation of a decreased tissue level of Iba1 across all examined regions of AD brain could be explained by reduced microglial cellularity or by loss of the Iba1 by a substantial fraction of microglia. A study of a large sporadic AD cohort found that a substantial fraction of AD microglia was negative for Iba1 and positive for MHCII, while the total number of microglial cells in fact did not differ between those with AD and control individuals.\textsuperscript{32} While Serrano-Pozo et al\textsuperscript{32} and Jay et al\textsuperscript{33} have proposed that the MHCII-positive/Iba1-negative cells represent peripheral myeloid cells that had acquired a microglial phenotype, it is also possible that resident AD microglia have lost Iba1 expression. We hypothesize that in the course of chronic activation, MHCII-positive/Iba1-positive cells may eventually express decreased levels of Iba1, a factor important for microglial functions, such as motility and phagocytosis.\textsuperscript{34} The idea of such reprogrammed microglia in AD\textsuperscript{35} is supported by several lines of evidence, such as a decline of essential microglial activities with progressive accumulation of β-amyloid plaques in the mouse model of AD\textsuperscript{36} and a strong negative correlation of β-amyloid burden and Iba1 level in the brains of patients with AD.\textsuperscript{37} A decreased Iba1 level is also found in the animal model of polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy.\textsuperscript{38} Thus, TREM2 R47H may exacerbate the dysfunctional phenotype of AD microglia by further reduction of Iba1.

Conclusions
The LOAD123 family reported herein exemplifies the complexity of the genetic landscape of LOAD, even in single pedigrees with an apparent autosomal dominant pattern of inheritance. In this pedigree, it is likely that ApoE4, TREM2 R47H, and rare variants in other genes, such as UNC5C D353N, are responsible for the marked occurrence of AD in the family. Our results also support the role of the TREM2 receptor in microglial clearance of aggregated proteins that is compromised in R47H carriers who may have shortened duration of disease.

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