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Missense variant in *TREML2* protects against Alzheimer's disease

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Abstract

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TREM and TREM-like receptors are a structurally similar protein family encoded by genes clustered on chromosome 6p21.11. Recent studies have identified a rare coding variant (p.R47H) in *TREM2* that confers a high risk for Alzheimer's disease (AD). In addition, common single nucleotide polymorphisms in this genomic region are associated with cerebrospinal fluid biomarkers for AD and a common intergenic variant found near the *TREML2* gene has been identified to be protective for AD. However, little is known about the functional variant underlying the latter association or its relationship with the p.R47H. Here, we report comprehensive analyses using whole-exome sequencing data, cerebrospinal fluid biomarker analyses, meta-analyses (16,254 cases and 20,052 controls) and cell-based functional studies to support the role of the *TREML2* coding missense variant p.S144G (rs3747742) as a potential driver of the meta-analysis AD-associated genome-wide association studies signal. Additionally, we demonstrate that the protective role of *TREML2* in AD is independent of the role of *TREM2* gene as a risk factor for AD.

Keywords: TREM2, Genome-wide association studies, Conditional analysis, Endophenotype, Gene, Alzheimer's disease, Association

1. Introduction

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Genome-wide association studies (GWAS) are a very powerful approach for identification of novel loci associated with disease status or other complex traits. However, these single nucleotide polymorphisms (SNPs) are usually not the functional variants driving the association and, in many cases, regional linkage disequilibrium (LD) prevents identification of a single candidate gene in the region. Often, additional studies are required to demonstrate unambiguously that the gene and/or variant implicated in disease risk is functionally related to pathogenesis.

Recently, the International Genomics of Alzheimer's Project (IGAP) identified 11 new loci ($p < 10^{-8}$) associated with risk for Alzheimer's disease (AD), and 13 additional suggestive loci (p value between 10^{-6} and 10^{-8}) ([Lambert et al., 2013](#)). Among the latter group, there is an inter-genic SNP (rs9381040; $p < 6.3 \times 10^{-7}$) located 5.5 Kb downstream from *TREML2* and 24 Kb upstream from *TREM2*. The *TREM* and *TREM-like receptor* genes clustered on chromosome 6p21.1 ([Ford and McVicar, 2009](#)) have different patterns of LD among them ([Cruchaga et al., 2013](#)). This genomic region has previously been implicated in genetic risk for AD ([Benitez et al., 2013](#), [Bertram et al., 2013](#), [Cruchaga et al., 2013](#), [Guerreiro et al., 2013](#), [Jonsson et al., 2012](#), [Reitz and Mayeux, 2013](#)). A low frequency missense variant in *TREM2* (p.R47H, minor allele frequency = 0.003) was reported to substantially increase risk for AD ([Benitez et al., 2013](#), [Guerreiro et al., 2013](#)). SNPs in this region

and 974 healthy elderly (age >70) control subjects with available age and gender data. Cases and elderly screened control subjects were recruited by the Medical Research Council (MRC) Genetic Resource for AD (Cardiff University; Institute of Psychiatry, London; Cambridge University; Trinity College Dublin), the Alzheimer's Research UK Collaboration (University of Nottingham; University of Manchester; University of Southampton; University of Bristol; Queen's University Belfast; the Oxford Project to Investigate Memory and Ageing, Oxford University); Washington University, St Louis, United States; medical research council PRION Unit, University College London; London and the South East Region AD project, University College London; Competence Network of Dementia, and Department of Psychiatry, University of Bonn, Germany; the National Institute of Mental Health AD Genetics Initiative. A number of 6129 control subjects were drawn from large existing cohorts with available GWAS data, including the 1958 British Birth Cohort (<http://www.b58cgene.sgul.ac.uk>), the KORA F4 Study and the Heinz Nixdorf Recall Study. All AD cases met criteria for either probable (National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association [NINCDS-ADRDA], Diagnostic and Statistical Manual of Mental Disorders [DSM-IV]) or definite (Consortium to Establish a Registry for Alzheimer's Disease [CERAD]) AD. All elderly controls were screened for dementia using the MMSE or ADAS-cog, were determined to be free from dementia at neuropathological examination or had a Braak score of 2.5 or lower. Genotypes from all cases and control subjects were previously included in the AD GWAS by [Harold et al. \(2009\)](#). Imputation of the dataset was performed using IMPUTE2 and the 1000 genomes (<http://www.1000genomes.org/>) Dec2010 reference panel (NCBI build 37.1). The imputed data was then analyzed using logistic regression including covariates for country of origin, gender, age, and 3 principal components were obtained with EIGENSTRAT (EIGENSOFT 4.2) ([Patterson et al., 2006](#)) software based on individual genotypes for the GERAD study participants.

2.5. European Alzheimer's disease initiative consortium

All AD cases were ascertained by neurologists from Bordeaux, Dijon, Lille, Montpellier, Paris, Rouen, and were identified as French Caucasian ([Dreses-Werringloer et al., 2008](#), [Group, 2003](#)). Clinical diagnosis of probable AD was established according to the DSM-III-R and NINCDS-ADRDA criteria. Control subjects were selected from the 3C Study ([Group, 2003](#)). This cohort is a population-based, prospective (7-years follow-up) study of the relationship between vascular factors and dementia. It has been carried out in 3 French cities: Bordeaux (southwest France), Montpellier (southeast France), and Dijon (central eastern France). A sample of non-institutionalized, over-65 subjects was randomly selected from the electoral rolls of each city. Between January 1999 and March 2001, 9686 subjects meeting the inclusion criteria agreed to participate. After recruitment, 392 subjects withdrew from the study. Thus, 9294 subjects were finally included in the study (2104 in Bordeaux, 4931 in Dijon, and 2259 in Montpellier). Genomic DNA samples 38 of 7200 individuals were transferred to the French Centre National de Génotypage. First stage samples that passed DNA quality control were genotyped with Illumina Human 610-Quad BeadChips (n = 452). At the end, we removed 308 samples because they were found to be first- or second-degree relatives of other study participants, or were assessed non-European descent based on genetic analysis using methods described in [Heath et al. \(2008\)](#). In this final sample, at 7 years of follow-up, 459 individuals suffered from AD with 97 prevalent and 362 incident cases. These AD cases were included as cases in the European Alzheimer's disease initiative (EADI) discovery dataset. We retained the other individuals as control subjects (n = 6017). The imputation was performed using 1000 Genomes multi-ethnic data (1000 G phase 1 integrated variant set release v3) as reference panel. Imputation was performed in 2 steps: pre-phasing with SHAPEIT (v2), followed by imputation with IMPUTE2. SNPs are used in the imputation process if call rate

>98%, Hardy-Weinberg equilibrium (HWE) p value > 1e-6, minor allele frequency (MAF) > 1.

2.6. CSF levels dataset

A description of the CSF dataset used in this study can be found in [Cruchaga et al. \(2013\)](#) and data included 1269 unrelated individuals recruited through the Knight-ADRC at Washington University (n = 501, 73% CDR = 0), the Alzheimer's Disease Neuroimaging Initiative (n = 394, 27% Clinical Dementia Rating [CDR] = 0), a biomarker consortium of Alzheimer disease centers coordinated by the University of Washington (n = 323, 61% CDR = 0), and the University of Pennsylvania (UPenn) (n = 51, 2% CDR = 0). Briefly, CSF tau, phospho-tau-181 (ptau), and amyloid beta ($A\beta_{42}$) levels were from research participants enrolled in longitudinal studies at the Knight-ADRC, ADNI, University of Washington, and University of Pennsylvania. CSF collection and $A\beta_{42}$, tau, and ptau181 measurements were performed as described previously ([Fagan et al., 2006](#)). The samples were genotyped using Illumina chips. Cases received a diagnosis of dementia of the Alzheimer's type, using criteria equivalent to the National Institute of Neurological and Communication Disorders and Stroke-Alzheimer's Disease and Related Disorders Association for probable AD ([McKhann et al., 1984](#)). Controls received the same assessment as the cases but were nondemented. All individuals were of European descent and written consent was obtained from all participants.

2.7. Statistical analyses

We performed multivariate logistic regression to evaluate the association between genetic markers and risk for LOAD adjusting for age, gender, population substructure, and study specific effects using PLINK (<http://pngu.mgh.harvard.edu/purcell/plink/>). Conditional analysis was performed to identify additional independent signals by conditioning on the top case-control GWAS hits. We first estimated the odds ratios for SNPs across cohorts. These models calculate crude odds ratios and confidence intervals from counts of heterozygous in case patients and control subjects in each study. Then we performed a fixed-effect model to combine the odds ratios from study-specific estimates into a summary measure. No multiple-testing correction was used in our analyses. The heterogeneity of effects was evaluated using Woolf test for heterogeneity ([Woolf, 1955](#)). Meta-analysis was conducted using the META package (<http://www.stats.ox.ac.uk/~jsliu/meta.html>) in R (version 3.0.1).

Association of CSF ptau with the genetic variants was analyzed as described previously ([Cruchaga et al., 2010](#), [Cruchaga et al., 2011](#), [Kauwe et al., 2011](#)). Briefly, CSF ptau values were log transformed to approximate a normal distribution. Because the CSF levels were measured using different platforms (Innotest plate ELISA vs. AlzBia3 bead-based ELISA, respectively), we were not able to combine the raw data. We extracted from the log-transformed value, the mean within each series for the log-transformation. No significant differences in the transformed CSF values of the different series were found. We used SAS (version 9.2) to analyze the association of SNPs with CSF biomarker levels. Age, gender, site, and the first 3 principal components were included as covariates. We also performed conditional analyses by including several variants in the model.

2.8. Genotyping

rs9381040 and rs3747742 were extracted from the GWAS data ([Cruchaga et al., 2013](#)), and confirmed by direct genotyping. The *TREM2*-p.R47H was genotyped using KASP genotyping assay (LGC Genomics), as previously described ([Benitez and Cruchaga, 2013](#), [Cruchaga et al., 2009](#), [Cruchaga et al., 2010](#), [Cruchaga et al., 2012](#)) on 2000 cases and control subjects from the Knight-ADRC.

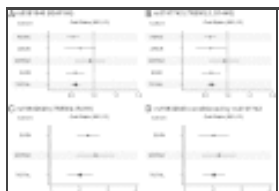
2.9. Cell-based analysis

Primary astrocytes and microglia were prepared from 2 litters (16 pups) of P1 C57BL/6 mice. Individual mice were pooled and 12 replicate co-cultures were plated in 25 cm² flasks. Co-cultures were treated with 0.2 ng/mL of mouse interleukin-1 beta (IL-1 β) (R&D 401-ML/CF) for 24 hours. Microglia was detached from the plate by shaking at 125 rpm for 1 hour in a 37 °C incubator. RNA was extracted using MiRNeasy mini kit (Qiagen 217004), according to manufacturer's instructions. The quantitative polymerase chain reaction assays for mouse Trem2 (ID: Mm04209424), Trem12 (ID: Mm01277362), and Saa3 (ID: Mm00441203) were obtained from Life Technologies (NY, USA).

3. Results

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Eight coding variants were validated in the *TREML2* gene ([Table 1](#)), which constitute the 53% (8/15) of the missense variants reported for *TREML2* gene in the Exome Variant server (release ESP6500SI-V2) for European Americans. Only 3 variants exhibit a MAF % higher than 1%: p.V25A (MAF = 5%), p.T129S (MAF = 4.5%), and p.S144G (MAF = 30%). Interestingly, according to our exome sequencing results all these variants are more common in control subjects than in AD cases, however they did not reach statistical significance with our whole-exome sequence sample size, although the three of them are more common in control subjects than AD cases ([Table 1](#)). Interestingly, the missense variant p.S144G (rs3747742) exhibited the highest LD ($r^2 = 0.73$, $D' = 0.86$) with the GWAS SNP, rs9381040 ([Table 1](#)), and the higher MAF among the validated missense variants in *TREML2*, which made it suitable for further analysis. Next, we performed a meta-analysis of the data from the ADGC, GERAD, EADI, and the Alzheimer's Research UK; studies (16,254 cases and 20,052 control subjects) we found that the minor alleles of both rs9381040 ($p = 1.21 \times 10^{-5}$; OR = 0.92, CI = 0.88–0.95), and rs3747742 ($p = 8.66 \times 10^{-5}$; OR = 0.93, CI = 0.89–0.96) reduce risk for AD ([Fig. 1](#), panel A and B). When rs3747742 is included in a logistic regression model as a covariate, rs9381040 is no longer significant ($p = 0.43$), and vice-versa, indicating that these SNPs are tagging the same signal. In addition, *TREM2*-p.R47H (rs75932628) was successfully imputed (imputation quality score information = 0.84 and 0.79) in the GERAD and EADI studies, and it displays a strong association with AD risk ($p = 1.3 \times 10^{-3}$; OR = 1.92, CI = 1.29–2.85) ([Fig. 1](#), panel C). When rs3747742 or rs9381040 are included as covariates in a conditional analysis, rs75932628 remains highly significant ($p = 1.27 \times 10^{-4}$ and $p = 1.19 \times 10^{-4}$, respectively) ([Fig. 1](#), panel D), suggesting that the *TREML2* and *TREM2* signals are independent from each other.



[Fig. 1](#)

Odds ratios for rs9381040 (IGAP hit), rs3747742 (*TREML2*, p.S144G), and rs75932628 (*TREM2*, R47H) among AD patients, as compared with control subjects, at each study center and overall. Shown are the combined estimates of the AD risk of possessing rs9381040 ...

We also performed a linear regression analysis for rs9381040 and rs3747742 with CSF levels of tau and ptau (n = 1269 individuals) ([Cruchaga et al., 2013](#)). rs9381040 ($p = 4.11 \times 10^{-4}$, beta = -0.02) and rs3747742 ($p = 1.4 \times 10^{-4}$, beta = -0.02) both exhibit a strong association with CSF ptau levels. The respective associations with CSF ptau are no longer significant when either SNP is included as a covariate in the conditional analysis. These results confirm via 2 independent datasets that the associations of rs9381040 and rs3747742 with CSF biomarker levels and with AD risk represent the

same signal. The *TREM2*-p.R47H variant was also genotyped in a subset of the CSF samples ($n = 835$). In these samples, 3 variants, rs9381040 ($p = 0.04$, $\beta = -0.02$) (Fig. 2, panel A), rs3747742 ($p = 0.02$, $\beta = -0.02$) (Fig. 2, panel B), and rs75932628 ($p = 0.0016$, $\beta = 0.2$) (Fig. 2, panel C) demonstrate a nominally significant association with CSF ptau levels. To determine whether the *TREM2* signal (rs3747742) is independent of *TREM2*-p.R47H, we removed all of the p.R47H carriers from the analysis. rs3747742 remained significantly associated with CSF ptau levels ($p = 0.03$) (Fig. 2, panel D). Furthermore, when *TREM2*-p.R47H was included in the model as a covariate for rs3747742 analysis, the association remained significant ($p = 0.02$), which suggests that the *TREM2* and *TREML2* signals are independent. Importantly, these associations confirmed the direction of the effect on CSF ptau levels: the minor allele of rs3747742 is associated with lower ptau levels ($\beta = -0.02$) and is predicted to be protective for AD risk (OR = 0.91; CI = 0.86–0.97), while the minor allele of *TREM2*-p.R47H is associated with an increased risk for AD (OR = 1.91, CI = 1.85–1.97) and higher levels of CSF ptau ($\beta = 0.2$).

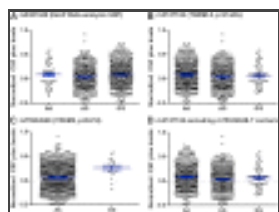


Fig. 2

Association of *TREM2* and *TREML2* variants with CSF ptau levels. Panel (A) CSF ptau181 levels by rs9381040 genotype (IGAP meta-analysis most significant SNP). AG + GG versus AA $p = 0.04$. (Panel B) CSF ptau181 levels by rs3747742 genotype ...

In addition, TREM and TREM-like receptors modulate the innate immune response by either amplifying or dampening Toll-like receptor-induced signals, playing critical roles in fine-tuning the inflammatory response (Ford and McVicar, 2009). TREM and TREM-like receptors demonstrate different patterns of expression and are likely to play different roles in the inflammatory response. To further understand the relative expression of *TREM2* and *TREML2*, we analyzed gene expression in primary mouse microglia and astrocytes stimulated by IL-1 β . Treatment of microglia with IL-1 β repressed expression of *TREM2* (Fig. 3, panel A), but increased expression of *TREML2* (Fig. 3, panel B). The opposing effects of this inflammatory cytokine on *TREM2* and/or *TREML2* expression is consistent with our genetic data and with evidence that *TREM2* and/or *DAP12* antagonizes inflammatory signaling in microglia while *TREML2* is not coupled to *DAP12* signaling and plays a pro-inflammatory role (Ford and McVicar, 2009).

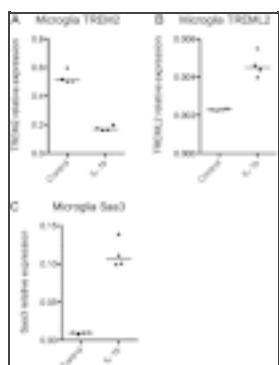


Fig. 3

Microglial expression of *TREM2* and *TREML2* show opposing effects in the presence of IL-1 β . *TREM2* panel (A) and *TREML2* panel (B) gene expression were analyzed in primary mouse microglia and astrocytes activated by 0.2 ng/mL IL-1 β for 24 hours. ...

4. Discussion

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In summary, these results demonstrate that the associations of missense variants in *TREM2* and *TREML2* with AD risk are independent. Moreover, our analyses suggest that the AD-associated GWAS signal is likely driven by the *TREML2* coding missense variant p.S144G (rs3747742); it results in a similar odds ratio to rs9381040. We also validated 2 other coding variants p.V25A and p.S129T in *TREML2* gene in moderate LD ($r^2 = 0.05$ and $D' = 1$) with the GWAS SNP, which both exhibited a

higher frequency among control subjects than in AD cases ([Table 1](#)). However, for both variants we only obtained data by whole-exome sequencing which limited our analysis about the role that these variants may play in the association of *TREML2* with AD risk. To prove that these additional variants are associated with AD risk we will need a larger sample size. Additionally, the purpose of this study was to find a functional coding variant in the *TREML2* gene that could explain the association for *TREML2* which was found in the recent IGAP meta-analysis. Our data suggest that there is a coding variant in *TREML2* that could explain the GWAS signal, but our data cannot rule-out of the presence of functional variants outside of the coding region.

We conclude that at least 2 genes in this gene cluster influence risk for AD: *TREM2*-p.R47H is associated with increased risk for AD (OR = 1.91, CI = 1.85–1.97) and *TREML2*-p.S144G is associated with reduced risk for AD (OR = 0.91; CI = 0.86–0.97). The mechanisms by which these variants influence AD risk are not currently understood, but it has been suggested that modulation of microglial activation might influence clearance of A β ([Benitez et al., 2011](#)). These results underline the importance of the inflammatory response in modulating risk for AD and suggest that other genes in this gene family may also harbor risk alleles for AD.

Disclosure statement

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The authors report no conflicts of interest.

All participants had agreed by signed informed consent to participate in genetic studies approved by our Institutional Review Board.

Acknowledgements

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