TYROBP genetic variants in early-onset Alzheimer's disease

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Abstract

We aimed to identify new candidate genes potentially involved in early onset Alzheimer's disease (EOAD). Exome sequencing was conducted on 45 EOAD patients with either a family history of Alzheimer's disease (AD, <65 years) or an extremely early age at onset (≤55 years) followed by multiple variant filtering according to different modes of inheritance. We identified 29 candidate genes potentially involved in EOAD, of which the gene *TYROBP*, previously implicated in AD, was selected for genetic and functional follow-up. Using three patient cohorts, we observed rare coding *TYROBP* variants in 9 out of 1110 EOAD patients, whereas no such variants were detected in 1826 controls (p-value=0.0001), suggesting that at least some rare *TYROBP* variants might contribute to EOAD risk. Overexpression of the p.D50_L51ins14 TYROBP mutant led to a profound reduction of TREM2 expression, a well-established risk factor for AD. This is the first study supporting a role for genetic variation in *TYROBP* in EOAD, with *in vitro* support for a functional effect of the p.D50_L51ins14 *TYROBP* mutation on TREM2 expression.

Keywords: Alzheimer's disease, TYROBP, TREM2, exome sequencing, burden test

1. Introduction

Alzheimer's disease (AD) is the most common cause of dementia affecting over 4 million individuals in the United States. A minority of patients have early-onset AD (EOAD, arbitrarily defined as an age at disease onset before 65 years of age) and a subset of those patients carry disease-causing mutations or duplications in one of three genes: *APP* (MIM #104760), *PSEN1* (MIM #104311) and *PSEN2* (MIM #606889). In the last few years, the use of exome sequencing has provided additional insights into the genetics of AD, identifying rare variants in *SORL1* (MIM #602005), *NOTCH3* (MIM #600276), *TTC3* (MIM #602259), *PLD3* (MIM #615698) and *TREM2* (MIM #605086) as potentially involved in the pathophysiology (Cruchaga, et al., 2014, Guerreiro, et al., 2013, Guerreiro, et al., 2012, Jonsson, et al., 2013, Kohli, et al., 2016, Pottier, et al., 2012). A large proportion of EOAD, however, remains unexplained due to paucity of large pedigrees for genetic screening. Although approximately 50% of EOAD patients are considered to be sporadic, we hypothesized that either variants compatible with autosomal recessive transmission or autosomal dominant transmission with reduced penetrance could be responsible for apparently sporadic EOAD patients. In order to identify candidate EOAD genes, we exome sequenced 45 EOAD patients from our Mayo Clinic EOAD cohort and applied bioinformatics filtering strategies using multiple modes of inheritance. Among the identified candidate genes, we chose to follow-up *TYROBP* because of its already reported upregulation in AD brains and its significant role in the

Abbreviations: AD, Alzheimer's diease; EOAD, early-onset Alzheimer's disease; LOAD, late-onset Alzheimer's disease; ADRC, Alzheimer's disease research center; CNR-MAJ, centre national de référence pour les malades Alzheimer jeunes; NINCDS-ADRDA , National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association; UTR, untranslated region; GATK, Genome Analysis Toolkit; VQSR, Variant Quality Score Recalibration; PCR, polymerase chain reaction; CMC, Combined and Multivariate Collapsing; MAF, minor allele frequency; NHD, Nasu-Hakola disease; HDLS, hereditary diffuse leukoencephalopathy with spheroids; CSF, cerebrospinal fluid; ALS, amyotrophic lateral sclerosis; FTD, frontotemporal dementia

pathogenesis of late-onset AD (LOAD) (Ma, et al., 2015, Zhang, et al., 2013). Moreover, TYROBP is the binding partner of TREM2 (Bouchon, et al., 2001), a well-established genetic risk factor for AD (MIM #107741) (Benitez, et al., 2013, Guerreiro, et al., 2013, Jin, et al., 2014, Jonsson, et al., 2013, Neumann and Daly, 2013, Pottier, et al., 2013, Ruiz, et al., 2014).

2. Materials and methods

2.1. Participants

Our primary patient series includes 597 EOAD patients which were ascertained from 1997 to 2015 at Mayo Clinic Jacksonville, Mayo Clinic Rochester, and the Mayo Clinic Brain Bank (Table 1). Patients were arbitrarily defined as EOAD if patients presented with a clinical diagnosis of AD at or before 65 years of age, or if patients were pathologically confirmed with AD at autopsy with an age at death before 70 years (irrespective of the onset age). Routine genetic screening of the known genes by Sanger sequencing in this EOAD cohort was performed and revealed 35 pathogenic *PSEN1* mutations, 8 *APP* mutations and duplications, 3 *PSEN2* mutations, 5 *MAPT* mutations, 2 *PGRN* mutations and 3 *C9ORF72* repeat expansions (Wojtas, et al., 2012). A total of 1036 white non-Hispanic healthy subjects of similar age and gender ascertained at Mayo Clinic Jacksonville were used as a control group (Table 1).

Additional EOAD patients (n=111), negative for pathogenic mutations in *APP*, *PSEN1* and *PSEN2* genes, were recruited in the Charles F. and Joanne Knight Alzheimer's Disease Research Center (ADRC) from Washington University, St Louis, USA (Supplementary Table 1). Additionally, 292 healthy individuals ascertained at Charles F. and Joanne Knight ADRC were used as control group (Supplementary Table 1).

Finally, a cohort of French EOAD patients (n=484), negative for pathogenic mutations in *APP*, *PSEN1* and *PSEN2* genes, were recruited over a 20-year period by the French National CNR-MAJ consortium. For comparison, a total of 498 controls were recruited in 5 different French cities and were all of French ancestry (Supplementary Table 1).

All patients were diagnosed according to the NINCDS-ADRDA (National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association) criteria (McKhann, et al., 2011). A comprehensive clinical examination including personal medical and family history assessment and neurological examination was performed on each patient. Patients were considered as having a positive family history if at least one first or second degree relative presented with a neurodegenerative disorder.

2.2. Standard protocol approvals, registrations, and patient consents

Written informed consent was obtained from patients and controls individuals for genetic analyses at all sites. Each study site obtained approval from the local ethics committee or institutional research board.

2.3. Exome sequencing analysis

Exome sequencing of 23 familial EOAD patients was performed as previously described (Guerreiro, et al., 2013). Exomes of the 22 sporadic EOAD patients were captured using the Agilent SureSelect Human All Exon V5 plus UTR kit. Sequencing was performed on an Illumina Genome HiSeq 2000 by 101-base paired-end reads. Alignment of sequence reads was performed against the Human Reference Genome build GRCh37. Variants were called using default settings from the GATK tool and following the best practices (McKenna, et al., 2010). Low-quality variants that did not pass the Variant Quality Score Recalibration (VQSR) filter (DePristo, et al., 2011) were removed. Genetic data from the two pilot studies were combined and analyzed using two independent pipelines in order

to maximize the chance of detecting potentially pathogenic variants. Specifically, filtering of variants was performed using the GEM.app application developed at the University of Miami (Gonzalez, et al., 2013) and using BioR developed at Mayo Clinic (Kocher, et al., 2014). All variants identified by at least one analysis pipeline were confirmed by Sanger sequencing.

2.4. Mutational analysis

For sequence validation, specific primers were designed surrounding each rare variant detected by Exome sequencing (Supplementary Table 2). In addition, all exonic regions of *TYROBP* were amplified by PCR using specific primers (sequences available upon request). Prior to sequencing, PCR products were purified using the AMPure system (Beckman Coulter Genomics, Brea, CA, USA). Sequence reactions were purified with CleanSEQ (Beckman Coulter Genomics, Brea, CA, USA) and then Sanger sequenced on an ABI3730xl Genetic Analyzer. Sequences were analyzed using Sequencher 4.8 software (Gene Codes Corporation, Ann Arbor, MI, USA).

2.5. Phase determination of p.G2E and p.V55L variants in *TYROBP*

Cerebellar RNA was extracted from patient A and a healthy subject using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) and quality was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), the RNA integrity number value obtained were above 8. cDNA was generated from 250ng of RNA from patient A and a healthy subject using the SuperScript® III First Strand Synthesis System kit (Invitrogen™, Carlsbad, CA, USA). A PCR was performed on the cDNA using a PCR

primer set with a reverse primer specific for the mutant T allele of the p.V55L variant (NM_003332.3:c.163G>T) (primers sequences available upon request) allowing us to Sanger sequence the nucleotides surrounding the p.G2E variant.

2.6. Cell culture transfection, mRNA expression and western blotting

HeLa cells were transiently co-transfected with: 1µg of human TREM2-cMyc and 1µg of either TYROBP-eGFP wild-type (TYROBP WT) or TYROBP-D50_L51ins14-eGFP mutant (TYROBP D50_L51ins14) plasmids. Control transfections were performed using either a co-transfection with human TREM2-cMyc and peGFP plasmids, or using a single transfection with 2µg of peGFP plasmid (Supplementary Material).

For mRNA expression analysis, transfected HeLa cells were harvested and total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). Reverse transcription reaction was then performed using the Superscript III system (Life Technologies, Carlsbad, CA, USA). Real-time quantitative PCR using an ABI7900 was performed in triplicate for each sample using gene expression probes for *TYROBP* (Hs00182426_m1), *TREM2* (Hs00219132_m1), *eGFP* (Mr04097229_mr), and *GAPDH* (Hs02758991_g1). Results were analyzed using SDS software version 2.2 (Life Technologies, Carlsbad, CA, USA) and quantifications of *TYROBP* and *TREM2* mRNA were first normalized to *GAPDH* levels and were determined relative to TYROBP WT transfected cells (Supplementary Material).

For western blotting, cells were harvested using radioimmunoprecipitation assay buffer (RIPA) (Boston Bioproducts, Ashland, MA). Equal volumes of cell lysate were run on 10–20% acrylamide tris-glycine gels (Invitrogen, Carlsbad, CA, USA). After transfer,

total TYROBP levels were detected using an eGFP antibody (MAB3580; Millipore, Darmstadt, Germany). TREM2 levels were assessed using a cMyc antibody (9E10; Roche, Penzberg, Germany). A GAPDH antibody was used as a protein loading control to visualize protein loading (Meridian Life Science, Memphis, ME, USA). Quantitative values from each blot were obtained using ImageJ 1.44 (National Institutes of Health, Bathesda, MD, USA). Numerical values of optical intensity were obtained by measuring the average pixel intensity per area for each band. Band intensity was then normalized to the total amount of protein using GAPDH. Protein expression level was assessed relative to the TYROBP WT condition (Supplementary Material).

2.7. Statistical analyses

Statistical analyses were performed using the Combined and Multivariate Collapsing test (CMC) using 100,000 permutation and the R software version 3.1.2 (2014-10-3, Vienna, Austria) (Li and Leal, 2008). Rare variants (minor allele frequency < 1%) affecting the TYROBP protein sequence were considered. CMC test collapses all rare variants and compares the proportions of variant carriers among cases and controls. No adjustment for age and gender were performed.

Statistical analyses for the western blot quantifications and the mRNA experiments were performed using a one-way ANOVA followed by Student t-tests for TREM2 and TYROBP levels using GraphPad Prism software (version 6.07 for Windows, GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Identification of potential candidate genes by exome sequencing

We performed two independent exome sequencing pilot studies on 45 white non-Hispanic EOAD patients selected from the overall Mayo Clinic cohort negative for mutations in the known genes: 23 patients with at least one affected relative with AD (mean age at onset: 56.7 years), and 22 patients with extreme young onset of EOAD (age at onset at or before 55, mean age at onset: 52.0 years) with no reported family history. Three different bioinformatic filtering strategies were subsequently employed to select candidate EOAD genes from our pilot sequencing dataset of 45 exomes (Table 2). First, we assessed our entire patient cohort (n=45) and selected heterozygous rare coding variants with a minor allele frequency (MAF) <0.1% in the NHLBI GO Exome Sequencing Project (ESP), Seattle, WA (URL: http://evs.gs.washington.edu/EVS/) based on the European cohort. Only variants affecting the protein sequence and shared between at least two individuals were selected (autosomal dominant model). Additionally, variants could not be present in more than 2 families in the GEM.app database. Using this strategy we were able to detect and validate 15 variants in 15 candidate genes (Table 2; Supplementary Table 2). Second, we focused on sporadic EOAD patients (n=22) and a potential recessive mode of inheritance. We selected all homozygous variants with a less stringent MAF of less than 5% in ESP European cohort that affected protein sequence, which resulted in the identification of 13 variants in 13 candidate genes (Table 2; Supplementary Table 2, homozygous model). Finally, in the same cohort of sporadic EOAD patients (n=22), we searched for compound heterozygous variants with a MAF<5% in the ESP European cohort that affected the protein sequences and were

present in at least two individuals. The variants identified were filtered out if present at the homozygous state in ExAC European population. This strategy revealed 4 validated variants in 2 candidate genes (Table 2, Supplementary Table 2, compound heterozygous model). Of note, variants were considered as compound heterozygous when at least two variants were found in the same gene.

3.2. Evaluation of *TYROBP* rare variants in the etiology of EOAD

Interestingly, the gene encoding *TYROBP* (MIM #604142*)* was identified using both the autosomal dominant and the compound heterozygote disease strategy, due to presence of variants c.5G>A (p.G2E) and c.163G>T (p.V55L) in two unrelated sporadic EOAD patients (patient A and patient B). Cerebellar brain tissue, available for patient A, allowed the extraction of mRNA and subsequent cDNA analysis, which demonstrated that both variants were inherited in *cis*, arguing in favor of a possible autosomal dominant mode of action for *TYROBP* variants in EOAD (Fig. 1A; Supplementary Material).

Given the strong functional link between TYROBP and AD, we expanded our genetic analysis of rare coding *TYROBP* variants in EOAD. We sequenced *TYROBP* in the entire EOAD Mayo Clinic cohort (n=597 including 43 other than white non-Hispanic patients and 56 mutation carriers) as well as in 1036 white non-Hispanic healthy subjects of similar age and gender ascertained at Mayo Clinic Jacksonville. This led to the identification of 4 additional rare variants (MAF<1%) affecting the coding region in 7 additional EOAD patients: c.67C>T (p.R23C), c.266C>T (p.S89L), c.151_152ins42 (p.Asp50_Leu51ins14), and c.238C>T (p.R80W) (Fig. 1B, Table 2, Table 3; Supplementary Table 3). No rare coding variants that affected the protein sequence were

found in healthy subjects. Notably, of the 9 EOAD patients carrying rare *TYROBP* variants, 3 patients also carried known AD gene mutations: the p.R80W variant was found twice in two Hispanic patients also harboring the pathogenic *PSEN1* c.617G>C (p.G206A) mutation; and the p.S89L variant was found in an *APP* duplication carrier. These patients were excluded from further statistical analysis. By collapsing rare coding variants with a MAF<1% and comparing the overall frequency of such variants between all white non-Hispanic EOAD patients without mutations in the known genes (n=6, representing 1.17% of 509 EOAD patients) and healthy subjects (n=1036; no mutation detected), we identified a significant enrichment in EOAD patients (p-value=0.002; CMC test).

To further substantiate this finding we examined two additional independent cohorts for the presence of rare coding variants in *TYROBP*. We studied 111 EOAD patients without mutations in the known AD genes and 292 healthy individuals from the Knight ADRC at Washington University by Sanger sequencing and we assessed exome sequencing data available on 484 French EOAD patients and 498 French controls without mutation in the known AD genes. Interestingly, we identified and confirmed by Sanger sequencing two rare coding variants in three unrelated French patients (Nicolas, et al., 2015), whereas no rare coding variants were detected in controls (n=595 EOAD patients and n=790 healthy subjects, Table 3, Supplementary Table 3). When combining these results, we observed a significant association between rare coding variants and EOAD: 6 rare coding *TYROBP* variants were identified in 9 unrelated patients out of 1110 white non-Hispanic EOAD patients as compared to an absence of such variants in 1826 healthy subjects (p-value=0.0001; CMC test). Of note, none of the patients harboring rare *TYROBP* variants

was homozygous for the *APOE* ε4 allele and the presence of the ε4 haplotype does not appear to have an effect on age at onset (Table 3, Supplementary Table 3).

3.3. *In vitro* functional analysis of TYROBP p.D50_L51ins14

To provide support for a functional effect of at least one of the identified *TYROBP* variants, we selected variant *TYROBP* p.D50_L51ins14 that overlaps with a critical amino-acid for the binding of TYROBP to TREM2 (D50). It was shown that an artificial mutation of amino-acid D50 reduced the ability of TYROBP to bind TREM2 and stabilize TREM2 C-terminal fragments resulting from γ-secretase cleavage (Wunderlich, et al., 2013,Zhong, et al., 2015). We identified the p.D50_L51ins14 mutation in a probable AD patient with an age at disease onset of 62 years. This patient showed a clear family history with a diagnosis of AD in the patient's father.

We co-expressed TYROBP p.D50_L51ins14 along with wild-type human TREM2 in HeLa cells in order to evaluate the impact of TYROBP p.D50_L51ins14 mutation on TYROBP and TREM2 levels. We found that the mutant form of TYROBP was expressed at a significantly lower protein level as compared to TYROBP WT (mean =0.30, SE=0.12; p-value=0.001, Student t-test; Fig. 2A and B), despite equal wild-type and mutant *TYROBP* mRNA expression (mean=0.93, SE=0.03; p-value=0.19, Student t-test; Fig. 2C). Moreover, TREM2 protein levels were significantly reduced in cells transfected with p.D50_L51ins14 mutant TYROBP as compared to cells transfected with wild-type TYROBP (mean=0.22, SE=0.02; p-value <0.0001, Student t-test; Fig. 2A and B). Interestingly, the reduction in TREM2 expression is also present at the mRNA level but to a far lesser extent than the protein level (mean=0.73, SE=0.02; p-value <0.0001,

Student t-test; Fig. 2C). Our control experiment in which we co-transfected TREM2 with eGFP (in the absence of TYROBP overexpression) also showed significantly reduced levels of *TREM2* mRNA (mean=0.66, SE=0.02; p-value <0.0001, Student t-test; Fig. 2C) and TREM2 protein (mean=0.18, SE=0.05; p-value <0.0001, Student t-test; Fig. 2A and B) similar to the decrease observed when co-expressed with the TYROBP mutant.

4. Discussion

In this study, we used an original bioinformatics filtering strategy to evaluate the impact of rare autosomal dominant and recessive variants in the genetics of EOAD and nominate potential candidate EOAD genes. We present a list of 29 candidate genes that could be considered in future genetics studies. We selected one gene from our list, *TYROBP*, based on its functional link to AD, and further characterized its potential involvement in EOAD. We first detected a nominally significant enrichment of rare coding *TYROBP* variants in EOAD compared to controls. Second, we identified one rare *TYROBP* variant with a profound effect on TREM2 expression in cell culture.

TYROBP was nominated as a candidate EOAD gene as part of our autosomal dominant and autosomal recessive (compound heterozygote) filtering strategies; however, brain mRNA and cDNA expression studies suggest that the observed variants were in cis, thereby favoring the hypothesis of an autosomal dominant inheritance pattern for *TYROBP* variants, possibly with reduced penetrance. *TYROBP* encodes a type I transmembrane protein consisting of a signal peptide sequence, a transmembrane segment and a cytoplasmic region containing an immunoreceptor tyrosine-based activation motif (ITAM; Fig. 1B). Within the central nervous system, *TYROBP* is preferentially expressed in microglia (Hickman, et al., 2013). Binding to its ligands, such as TREM2, leads to phosphorylation of TYROBP's tyrosine residues (positions 91 and 102) and recruitment of downstream proteins, which regulates pro-inflammatory responses and phagocytosis (Malik, et al., 2015, Paradowska-Gorycka and Jurkowska, 2013, Zhong, et al., 2015). In addition to its major role in immune system regulation and phagocytosis, TYROBP might be involved in Aβ turnover (Zhang, et al., 2013). Similar

to *TREM2* mutations, loss-of-function mutations in *TYROBP* have already been described in the recessively inherited Nasu-Hakola disease (NHD, MIM #221770) (Paloneva, et al., 2000), a rare condition characterized by systemic bone cysts and dementia. Moreover, TYROBP regulates macrophage proliferation through CSF1R (MIM #164770), which is encoded by a gene previously implicated in hereditary diffuse leukoencephalopathy with spheroids (HDLS, MIM #221820), a white-matter disease clinically characterized by behavioral, cognitive and motor abnormalities (Rademakers, et al., 2012). The mechanism leading to cognitive decline in these diseases is still under investigation; however, partial loss of the CSF1R/TYROBP signaling pathway has been suggested to be responsible for the neurological phenotypes observed in NHD and HDLS.

In our study, we detected an enrichment of *TYROBP* rare coding variants in EOAD compared to controls. We acknowledge that the level of significance did not reach the threshold of exome-wide significance ($2.5x10^{-6}$ for Bonferroni correction accounting for ~20,000 genes); however, we argue that our results are at least suggestive of a subset of *TYROBP* variants impacting EOAD disease risk. Remarkably, in line with this hypothesis, overexpression of TYROBP p.D50_L51ins14 leads to a loss of TYROBP protein and an almost total loss of TREM2 protein expression in cell culture. This reduction in TREM2 protein levels was partially explained by a 30% loss in *TREM2* mRNA expression but other mechanisms such as destabilization of TREM2 protein may also be involved. In either way, the strong reduction in TREM2 levels when co-expressed with mutant *TYROBP* suggests that, based on the current cellular model, the p.D50_L51ins14 mutation leads to a loss of TYROBP expression and/or function and reduced TREM2 levels. In this respect it is important to note that a decrease in soluble

TREM2 expression has been reported in cerebrospinal fluid (CSF) of patients harboring heterozygous variants in *TREM2*, which are known to lead to NHD in the homozygous state (Piccio, et al., 2016). Additionally, other rare variants in *TREM2* that have been reported as risk factors for AD correlate with a lower abundance of TREM2 in the CSF. Our results for the p.D50_L51ins14 mutation align with those findings and reinforce the idea that a loss of TREM2/TYROBP function might contribute to the physiopathology of AD. Whether and how the other 5 observed *TYROBP* rare variants affect TYROBP biology remains to be studied. Two of the *TYROBP* variants (p.G2E and p.R23C) are located in the signal peptide segment of TYROBP and might affect TYROBP localization or processing. Moreover, variant p.V47A is located in close proximity to p.D50_L51ins14 and might similarly affect the TREM2-TYROBP interaction and TREM2 stabilization. Finally, we note that we also detected rare coding *TYROBP* variants in 3 EOAD patients already harboring pathogenic mutations in *APP* and *PSEN1*. It remains possible that these *TYROBP* variants contribute to the disease in these patients, especially in light of the oligogenic disease mechanism that is gaining attention in other neurodegenerative disorders such as amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) (van Blitterswijk, et al., 2012).

In addition to *TYROBP*, we provide a list of 28 additional potential EOAD genes. Two notable candidates, which we have not yet followed-up genetically, are *AMPH* (MIM #600418*)* and *CD300E* (MIM #609801). Specifically, we identified in two unrelated individuals a non-sense variant in *AMPH* which encodes the amphiphysin-1 protein involved in synaptic endocytosis (Wu, et al., 2009). AMPH1 has been shown to be reduced in frontal and temporal cortex of AD patients as well as in the JNPL3 tauopathy

mouse model (De Jesus-Cortes, et al., 2012), suggestive of a role in tau-mediated neurodegeneration. Interestingly, amphiphysin-2, also known as *BIN1* (MIM #601248), resides at one of the most significant genome-wide association loci for LOAD risk (Lambert, et al., 2013). We also detected a non-sense variant in *CD300E*, which was present in two unrelated individuals. CD300E is a type I transmembrane protein and was shown to interact with *TYROBP* (Brckalo, et al., 2010). Whether *AMPH*, *CD300E* or any of the other candidate genes identified in our study are implicated in EOAD will require systematic mutation and association analyses in large EOAD and control cohorts. Similarly, in the near future, large scale on-going sequencing projects focused on LOAD may allow evaluating the impact on LOAD risk of the candidate genes identified in the current study.

5. Conclusions

In summary, we propose the use of extreme phenotypes such as very young EOAD patients or EOAD patients with a family history, as well as the use of multiple prioritization strategies as an efficient strategy to identify candidate genes for neurodegenerative disorders. We further present, for the first time, an association of rare coding variants in *TYROBP* with EOAD, which adds to the growing body of evidence suggesting that TYROBP is an important player in the physiopathology of AD.

Disclosure statement

The authors declare have no conflicts of interest to disclose. The ethics committees of all participating institutions have approved this study.

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Appendix A. Supplementary data

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TABLES

Table 1. Mayo Clinic EOAD and control cohort characteristics

	Mean age at onset in years (range)	**Mean age at death in years** (range)	**Mean age last visit in years** (range)	**% females** (n)	**% White non-Hispanic** (n)	**% Hispanic** (n)	**% Black or African American** (n)	**% Asian** (n)	**% Mutation carriers** (n)
EOAD patients (*n*=597)	55.93 (25–67)	63.95 (36-79)	NA	49.4 (295)	92.8 (554)	2.7 (16)	4.0 (24)	0.5 (3)	8.7 (52)
Controls (n=1036)	NA	NA	75 (27-99)	53.0 (549)	100.0 (1036)	NA	NA	NA	NA

Key: EOAD, Early Onset Alzheimer's Disease; n, number; NA, Not Applicable

Table 2. Strategies used to identify candidate EOAD genes in exome sequencing data

Strategy	Autosomal Dominant	Autosomal Recessive	
Coding changes	Missense/Stop-gain/Stop-loss/Splice/Indels/Start loss and gain	Missense/Stop-gain/Stop-loss/Splice/Indels/Start loss and gain	Missense/Stop-gain/Stop-loss/Splice/Indels/Start loss and gain
Number of EOAD patients	n=45	n=22	n=22
Variant types	Heterozygous variants	Homozygous variants	Compound heterozygous variants
ESP European American MAF*	<0.1%	<5%	<5%
HapMap European MAF*	<0.1%	<5%	<5%
Additional filters	Same variant in two unrelated individuals No more than 2 families in the GEM.app** database with the same variant	No additional filter	Same variant in at least two unrelated individuals No homozygotes in ExAC European***
Number of candidate genes	15	13	2

Key: EOAD, Early Onset Alzheimer's Disease; n, number of patients included

*Minor allele frequency (MAF) from the Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA (URL: http://evs.gs.washington.edu/EVS/)

**Filtering was done on allele count from the GEnomes Management Application (GEM.app) database, regrouping exome sequencing from 2200 families (September 2014).

***Filtering was done on Broad Institute Exome Aggregation Consortium (ExAC) genotypes.

Table 3. Non-Hispanic whites without mutations in known AD genes included in the statistical analyses

Site	Patients		Healthy individuals	
	TYROBP mutation carriers	Non mutation carriers	*TYROBP* mutation carriers	Non mutation carriers
Mayo Clinic*	6	509	0	1036
Knight ADRC	0	111	0	292
France	3	481	0	498
Total**	9	1101	0	1826

*: pvalue=0.002

**: pvalue=0.0001

FIGURES LEGENDS

Fig. 1. Molecular genetic analysis of *TYROBP*.

(A) Panel A includes a chromatogram of several bases surrounding the p.G2E variant (red square) of patient A and an healthy subject. (B) Schematic representation of TYROBP protein along with its different domains and the rare coding variants identified in the overall EOAD cohort. A PCR was performed on the cDNA from patient A using a PCR primer set with a reverse primer specific for the mutant T allele of the p.V55L variant (NM_003332.3:c.163G>T) allowing us to specifically amplify the chromosome carrying the c.163T allele. This PCR fragment also spanned the sequence surrounding the p.G2E (NM_003332.3:c.5G>A) variant. Sanger sequencing of the PCR product showed the mutant A allele of the p.G2E variant suggesting that the variant is in cis with the p.V55L variant.

Fig. 2. Effect of TYROBP mutant p.D50_L51ins14 on TREM2 expression.

(A) Co-expression of TYROBP mutant with TREM2 decreases the level of TREM2 protein expression. Western blot of HeLa cells transfected with TREM2-Myc in combination with wild-type TYROBP-eGFP (TYROBP WT-eGFP) or with the p.D50_L51ins14 (TYROBP mutant-eGFP) or a vector control eGFP. The levels of TYROBP and TREM2 were visualized using an eGFP or cMyc antibody, respectively. GAPDH was used as loading control. (B) Quantification of TYROBP and TREM2 protein levels in HeLa cells transfected as described in (A). Quantification is assessed relative to the TYROBP WT-eGFP transfection condition after normalization to GAPDH levels. (C) Quantification of mRNA levels in HeLa cells transfected as described in (A). **: $p<0.01$; ***: $p<0.0001$, Student t-test on $n\geq4$ replicates.

Figure(s)

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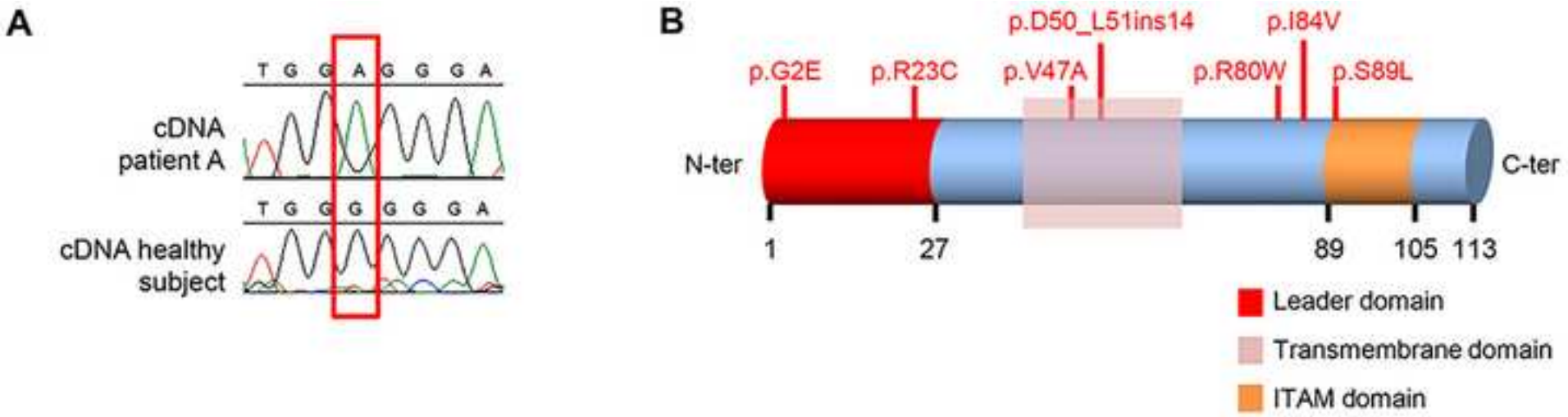

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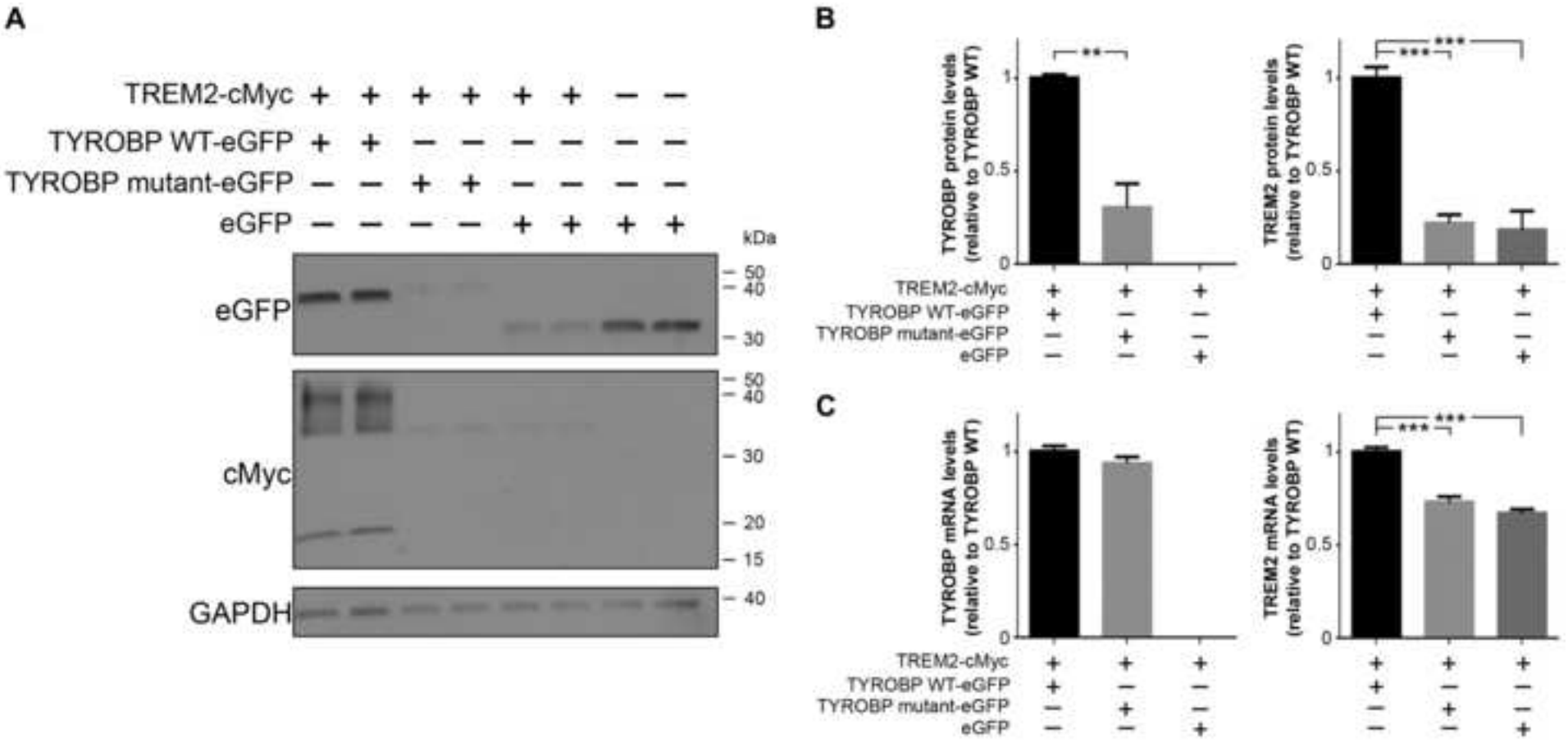

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Highlights.

- Exome sequencing of highly selected EOAD patients nominates 29 candidate genes
- Rare coding variants in TYROBP are genetically associated with EOAD
- TYROBP p.D50_L51ins14 is a potentially pathogenic variant altering TREM2 levels