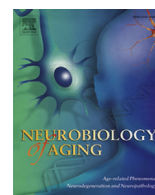




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EIF2AK3 variants in Dutch patients with Alzheimer's disease

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ABSTRACT

Next-generation sequencing has contributed to our understanding of the genetics of Alzheimer's disease (AD) and has explained a substantial part of the missing heritability of familial AD. We sequenced 19 exomes from 8 Dutch families with a high AD burden and identified *EIF2AK3*, encoding for protein kinase RNA-like endoplasmic reticulum kinase (PERK), as a candidate gene. Gene-based burden analysis in a Dutch AD exome cohort containing 547 cases and 1070 controls showed a significant association of *EIF2AK3* with AD (OR 1.84 [95% CI 1.07–3.17], *p*-value 0.03), mainly driven by the variant p.R240H. Genotyping of this variant in an additional cohort from the Rotterdam Study showed a trend toward association with AD (*p*-value 0.1). Immunohistochemical staining with pPERK and pelf2α of 3 *EIF2AK3* AD carriers showed an increase in hippocampal neuronal cells expressing these proteins compared with nondemented controls, but no difference was observed in AD noncarriers. This study suggests that rare variants in *EIF2AK3* may be associated with disease risk in AD.

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1. Introduction

Alzheimer's disease (AD) is the most common cause of dementia, characterized by progressive decline in memory and other cognitive functions (Ballard et al., 2011). Genetic factors are strongly linked to AD, and in about 5% of cases, an autosomal dominant mode of inheritance has been reported (St George-Hyslop, 1999). In autosomal dominant forms of early-onset AD, mutations in β -amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*), and presenilin 2 (*PSEN2*) have been found to be causative genes (Goate et al., 1991;

Levy et al., 1990; Levy-Lahad et al., 1995; Rogaev et al., 1995; Sherrington et al., 1995); this accounts for approximately 13% of early-onset AD (Campion et al., 1999). In late-onset AD, the $\epsilon 4$ allele of apolipoprotein E gene has been found to be the most common risk factor (Farrer et al., 1997).

Neuropathologically, the aggregation of misfolded proteins is a major hallmark of many neurodegenerative disorders (Hetzel and Mollereau, 2014). The accumulations of extracellular amyloid plaques and intracellular neurofibrillary tangles are the hallmarks of AD (Braak and Braak, 1991). Previous studies suggest that disrupted protein homeostasis in the endoplasmic reticulum (ER) and activation of unfolded protein response (UPR) may be major drivers in AD pathogenesis (Hetzel and Mollereau, 2014; Scheper and Hoozemans, 2015). The UPR is induced by 3 transmembrane proteins in the ER: protein kinase RNA-like endoplasmic reticulum

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kinase (PERK), inositol regulating enzyme 1 (IRE1), and activating transcription factor 6 (ATF6). Activation of UPR led to transient suppression of protein synthesis, and increased expression of genes aimed to restore the homeostasis of the ER (Hetz and Mollereau, 2014). Pharmacological and genetic manipulation of the UPR pathways in animal studies, in particularly the PERK pathway, has been reported to inhibit neurodegeneration (Smith and Mallucci, 2016).

Advances in next-generation sequencing technology have contributed substantially to our understanding of the genetics of AD. In recent years, studies using whole-exome sequencing (WES) and whole-genome sequencing reported the association of rare variants in *PLD3*, *ABCA7*, *TREM2*, and *SORL1* with an increased risk in AD (Cruchaga et al., 2014; Cuyvers et al., 2015; Guerreiro et al., 2013; Holstege et al., 2017; Pottier et al., 2012). Furthermore, a large exome microarray study identified rare coding variants in *PLCG2*, *ABI3*, and *TREM2*, explaining a small part of missing heritability in AD (Sims et al., 2017). These studies indicate the existence of other rare variants related to the heritability of AD.

In this article, we performed WES in 8 Dutch AD families with probable autosomal dominant inheritance and identified eukaryotic translation initiation factor 2 alpha kinase 3 (*EIF2AK3*), encoding for PERK, as a candidate AD risk gene in 2 of these families. Together with previous reports on an increased activation of PERK in AD brains and the involvement of PERK in memory and learning (Rozpedek et al., 2015), these findings suggest the possible role of *EIF2AK3* in the pathogenesis of AD.

2. Materials and methods

2.1. Subjects

Our discovery data set included 19 AD patients from 8 Dutch families with a high AD burden. Each family had at least 2 patients with AD suggestive of an autosomal dominant inheritance pattern, except 1 family with an uncertain mode of inheritance due to the early death of both parents. The mean age at disease onset in the families varied from 62.5 to 71.3 years (Table 1). Nondemented first- and second-degree family members of each family were also included if available. Using WES, all patients were screened negative for mutations in *PSEN1*, *PSEN2*, and *APP*; *APP* copy number mutations were also excluded. For WES, we included DNA samples of at least 2 patients with AD from each family. Nondemented family members with a minimum age of 65 years were used to test for segregation in their respective family.

Patients and family members were recruited after referral to the Department of Neurology in the Erasmus Medical Center or after visiting (nursing) homes. Diagnosis of probable AD was confirmed

in all patients according to the National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer's Disease and Related Disorders Association criteria for AD (McKhann et al., 2011).

To replicate the association of our candidate gene with AD, we used exome data available from 547 AD cases and 1070 controls from 3 different sites (the Rotterdam Study, Amsterdam Dementia Cohort [ADC-VUmc], and Alzheimer Centrum Erasmus MC [AC-EMC]) included from a Dutch AD exome data set, previously described by Holstege et al. (Holstege et al., 2017). We then genotyped our candidate variant in 1055 AD cases and 6162 controls from the Rotterdam Study (Ikram et al., 2017); any individuals from the Rotterdam Study included in the exome data were excluded for genotyping.

Our study has been approved by the Medical Ethical Committee of Erasmus Medical Center, and written informed consent was obtained from all participants or their legal representatives.

2.2. Whole-exome sequencing analysis

Exomes of 19 AD patients from the discovery set, the Rotterdam Study cohort, and the AC-EMC cohort were captured using the NimbleGen SeqCap EZ Exome Capture Kit v2. Exomes from the ADC-VUmc cohort were captured using the NimbleGen SeqCap EZ Exome Capture Kit v3. All data were generated at the Human Genomics Facility (HuGeF; www.glimdna.org) at Erasmus MC Rotterdam, the Netherlands. DNA from each sample was prepared using the Illumina TruSeq Paired-End Library Preparation Kit, and 100-bp paired end reads were acquired by sequencing the libraries on a HiSeq 2000. For the Dutch exome data set, we used the overlapping regions between capture kits during calling of the data. Sequencing reads were aligned to the hg19 human genome assembly using BWA-MEM (version 0.7.3a) (Li and Durbin, 2009), and Picard Tools (version 1.9) (Li et al., 2009) were used to mark duplicates and to sort the alignments. Subsequently, Genome Analysis Toolkit (GATK) (version 3.3) was used to perform indel realignment and base quality score recalibration (McKenna et al., 2010). Haplotype-Caller from GATK was used to create genomic VCF files and to call variants from these genomic VCF files. For the exome data from the 8 families (discovery set), we used hard filters according to GATK best practices to filter out low-quality variants. For the exome data from the 3 Dutch cohorts, we used variant quality score recalibration with >99% sensitivity to filter out low-quality variants. Subsequently, Plink was used to calculate principal components (PCs), and outliers on the first 2 PCs were removed (Purcell et al., 2007). Related individuals with identity-by-descent value > 0.1 were also removed from the analysis set. All individuals in the WES data were checked for sex concordance using Plink (Purcell et al., 2007).

Table 1
Baseline characteristics of the families

Family	Cases	Controls	WES cases	Mean age at onset (range)	Mean age at last visits of controls (range)	% Female	APOE fraction $\epsilon 2/\epsilon 3/\epsilon 4$
NLAD 1	5	8	3	70.4 (60–89)	69.1 (65–77)	46.2	0.2/0.5/0.3
NLAD 2	2	2	2	62.5 (52–73)	69.0 (68–70)	50.0	0/0.25/0.75
NLAD 3	5	2	3	71.3 (68–77)	78.5 (71–86)	71.4	0/0/1
NLAD 4	5	1	2	62.8 (59–65)	66.7 (61–72)	57.1	0/0.12/0.88
NLAD 5	2	0	2	66.0 (NA)	NA	0.0	0/0.75/0.25
NLAD 6	3	1	3	67.7 (64–70)	69.0 (NA)	75.0	0/0.67/0.33
NLAD 7	2	3	2	71.0 (66–76)	73.3 (69–78)	20.0	0/0/1
NLAD 8	2	4	2	64.5 (59–70)	71.4 (70–73)	50.0	0/1/0

The number of patients and controls included from each family. Cases are the total number of included patients with Alzheimer's disease and patients with mild cognitive impairment. Controls contain the total number of included individuals without subjective of objective memory impairment during the last visit. Age at onset is the mean age of first disease onset of all included cases, and the age at last visits is the mean age of all included controls. Age at onset and age at last visits in years.

Key: AD, Alzheimer's disease; NA, not available; WES, whole-exome sequencing.

Variants from all data sets were annotated using ANNOVAR (Wang et al., 2010).

In our discovery set, we used a family-based analysis to identify candidate genes from the 8 families. Each family was analyzed separately to identify the candidate variants in their respective family. We focused on shared variants among the affected family members, which resulted in an amino acid change. Subsequently, variants with a frequency of 0.5% or lower in 1000 genomes, NHLBI Exome Sequencing Project (ESP), Exome Aggregation Consortium (ExAC), Genome of the Netherlands, and in-house WES data from the Rotterdam Study were selected (Supplementary Table 1) (Genome of the Netherlands, 2014; Genomes Project et al., 2015; Lek et al., 2016; Tennessen et al., 2012; van Rooij et al., 2017). If the same variant or different variants in the same gene were identified in at least 2 families, these variants were selected as candidates for follow-up and tested with Sanger sequencing for segregation in their respective families.

2.3. Sanger sequencing

We used Primer 3 (Untergasser et al., 2012) to design primers for candidate variants. PCR amplification was performed using Qiagen Taq DNA polymerase (Qiagen, CA, USA). Direct sequencing of PCR products was performed using Big Dye Terminator chemistry ver. 3.1 (Applied Biosystems) and run on an ABI3130 genetic analyzer and an ABI3730xl genetic analyzer (Applied Biosystems, CA, USA). The sequences were analyzed with Sequencher software, version 4.5 (Genecodes, VA, USA), and SeqScape, version 2.6 (Applied Biosystems).

2.4. Genotyping of rs147458427 variant in EIF2AK3

The variant rs147458427 (p.R240H) was genotyped using TaqMan SNP Genotyping Assays, and genotypes of rs147458427 were determined using TaqMan Allelic discrimination. Signals were read with the TaqMan 7900HT (Applied Biosystems Inc) and analyzed using the Sequence Detection System 2.4 software (Applied Biosystems Inc). To evaluate genotyping accuracy, all heterozygous calls were typed twice to confirm genotypes. Single-variant association effects for AD association were calculated using R (version 3.2.3) “seqMeta” tool v.1.6.0 adjusting for gender. APOE status was added as a covariate in the secondary analysis.

2.5. Statistical analysis of the candidate genes in the Dutch exome data set

Single-variant association effect for AD association was calculated using R (version 3.2.3) “seqMeta” tool v.1.6.0 adjusting for gender. Burden test was calculated for our top candidate gene in the family-based analysis using *burdenMeta* function in “seqMeta” tool v.1.6.0. Only variants with minor allele frequency (MAF) $\leq 1\%$ in ExAC were included in the burden test, adjusting for gender. In the secondary analysis, we performed these analyses on our top candidate gene, adjusting for gender and APOE status.

2.6. Histology and immunohistochemistry

The Netherlands Brain Bank performed brain autopsy according to their Legal and Ethical Code of Conduct. Tissue blocks of 3 EIF2AK3 carriers (2 from family NLAD 1 and 1 from family NLAD 4) were taken from all cortical areas, hippocampus, amygdala, basal ganglia, substantia nigra, pons, medulla oblongata, cerebellum, and cervical spinal cord. They were embedded in paraffin blocks and subjected to routine staining with hematoxylin and eosin, periodic acid–Schiff reaction, and silver staining. Immunohistochemistry was

performed with antibodies directed against phosphorylated pancreatic endoplasmic reticulum kinase (pPERK) (sc-32577; Santa Cruz biotechnology, CA; 1:12,800) and phosphorylated eukaryotic initiation factor-2 α (pelf2 α) (SAB4504388; Sigma-Aldrich, St. Louis, MO; 1:100). We performed staining of pPERK and pelf2 α on the frontal, temporal, and hippocampal regions of our 3 pathological-confirmed AD EIF2AK3 carriers, 3 AD noncarriers, and 3 non-demented controls. Immunohistochemical staining of the neurons with pPERK and pelf2 α was scored with a semiquantitative method using a modified version of the scale developed by Stutzbach et al. and Hoozemans et al.: Negative (–): no cells stained, rare (+): 1–3 cells stained, ++: 4–20 cells stained or up to 10 percent of cells stained, +++: 20+ cells stained or 11 to 30 percent of cells stained, and ++++: high density of stained cells (>30 percent) in almost every field of the section (Stutzbach et al., 2013) (Hoozemans et al., 2009). In the frontal and temporal regions, the average number of positive stained cells per field was counted in 9 different fields of the cortical layer at 20x magnification. In the hippocampus, we used a different scoring method as this region is often severely affected in AD with extensive neuronal loss. We counted the total number of neurons with a nucleus, as well as the number of these neurons containing pPERK or pelf2 α staining to calculate the percentage of stained neurons. We focused on the cornu ammonis 1 (CA1) and subiculum, as these contain the largest number of positive stained cells and calculated the average percentage of stained cells per field in 3 different fields of CA1 and subiculum, each at 40x magnification.

We used Mann-Whitney *U* test to examine the difference between AD EIF2AK3 carriers and noncarriers. All tests are 2-sided significant tests, and a *p*-value below 0.05 was assumed as being statistically significant.

2.7. Immunoblot analysis

Postmortem fresh-frozen brain tissue of the frontal cortex from 3 carriers of EIF2AK3 mutations (III:15 and III:18 from family NLAD 1, and III:7 from family NLAD 4, Supplementary Fig. 1) and 3 AD cases were extracted with buffers of increasing strength (Neumann et al., 2006). Briefly, gray matter was extracted at 5 mL/g (volume/weight) using low salt buffer (10 mM Tris, pH 7.5, 5 mM EDTA, 1 mM DTT, 10% sucrose, and a cocktail of protease inhibitors), high salt-Triton buffer (low salt + 1% Triton X-100 + 0.5 M NaCl), myelin floatation buffer (30% sucrose in low salt + 0.5 M NaCl), and sarkosyl (SARK) buffer (1% N-lauroylsarcosine in low salt + 0.5 M NaCl). The SARK insoluble material was extracted in 0.25 mL/g urea buffer (7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate {CHAPS}, 30 mM Tris, pH 8.5). Proteins were resolved by 7.5% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). Following transfer, membranes were blocked with Tris-buffered saline containing 3% powdered milk and probed with the antibody p-PERK (sc-32577; Santa Cruz). Primary antibodies were detected with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch), and signals were visualized by a chemiluminescent reaction (Millipore) and the Chemiluminescence Imager Stella 3200 (Raytest).

3. Results

3.1. Family-based exome analysis of the discovery set

In our discovery analysis of 19 AD patients from 8 families, we found an average of 91 (range 26–136) candidate variants per family after filtering (Supplementary Table 1). Combining the candidate variants of the 8 families, we found 101 variants in 36 candidate genes, with some genes showing many variants shared

among families (Supplementary Table 2). We excluded the *MUC* genes as potential candidates as these are reported as frequent hitters in many WES data sets (Fuentes Fajardo et al., 2012). We selected the gene *EIF2AK3*, encoding for pancreatic endoplasmic reticulum kinase (PERK) as a top candidate gene (Sherrington et al.), based on its involvement in memory and learning, and on its neurodegenerative role in AD and other neurodegenerative diseases (Ohno, 2018; Scheper and Hoozemans, 2015).

The first *EIF2AK3* variant, p.R240H (rs147458427), was heterozygous in 4 affected individuals (including 1 with mild cognitive impairment) of family NLAD 1, and in 1 nondemented, 72-year-old cousin of the proband (Supplementary Fig. 1). This variant had a CADD score of 31 and a frequency of 8.00×10^{-4} in ExAC. The second *EIF2AK3* variant, p.N286S (rs150474217), had a low CADD score of 0.002 and a frequency of 3.00×10^{-5} in ExAC and was confirmed in 4 patients with AD from family NLAD 4 and in 1 nondemented, 72-year-old individual at last visit. One sibling with memory complaints and a normal Mini-Mental State Examination score did not carry the variant. Two of three patients with AD in family NLAD 4 carried homozygous *APOE* $\epsilon 4$; the third patient was heterozygous for *APOE* $\epsilon 4$. All patients were diagnosed with early-onset AD.

Sanger sequencing on the remaining variants in the 32 candidate genes shared among the 8 families (*MUC* genes excluded) confirmed variants in 15 genes (Supplementary Table 2). Segregation analysis of the variants in these 15 genes in their respective family did not show perfect segregation for most variants; the segregation in some variants could not be tested due to limited samples from related individuals.

3.2. Evaluation of *EIF2AK3* variants in Dutch cohorts

To determine the genetic association of *EIF2AK3* in AD, we performed gene-based burden analysis of *EIF2AK3* variants on the Dutch AD WES data set. We detected 23 *EIF2AK3* variants in this data set (Fig. 1 and Supplementary Table 3), of which 19 had an allele frequency <1% in ExAC; 17 of these rare variants were missense mutations. Burden test of all variants in *EIF2AK3* with MAF <1% in ExAC showed an increased risk for AD (OR = 1.84; 95% CI 1.07–3.17, $p = 0.03$). Single-variant analysis showed more carriers of variant p.R240H in cases (OR = 4.22; 95% CI 1.06–16.80, $p = 0.04$), but the nominal significant difference did not sustain the Bonferroni correction (Supplementary Table 3). We then performed a second analysis with *APOE* as an additional covariate showing the frequency of *EIF2AK3* carriers with at least one copy *APOE* $\epsilon 4$ is 62% (16/26). The single-variant analysis of p.R240H (OR=4.47, $p = 0.04$)

and the burden analysis (OR = 1.9, $p = 0.025$) were similar to the analysis without *APOE* as a covariate.

As the variant p.R240H showed a suggestive signal with a high CADD score, we genotyped this variant in an independent cohort from the Rotterdam Study containing 1055 cases and 6162 controls. We found an increased frequency in AD cases compared with controls (OR = 3.03; 95% CI 0.78–11.48, $p = 0.10$), and an association with AD after adjusting for *APOE* as an additional covariate (OR = 2.57; 95% CI 0.69–9.51, $p = 0.16$); however, in both cases, the results were not statistically significant.

3.3. Immunohistochemistry and immunoblot analysis

In our *EIF2AK3* carriers, many neurons with positive staining for pPERK and pelf2 α were seen in the hippocampus, as well as a low to moderate number of positively stained neurons in the frontal and temporal cortex (Table 2). The activated pPERK and pelf2 α staining in neurons were punctate shaped and were located in the cytoplasm, as reported in previous studies (Fig. 2A–F) (Hoozemans et al., 2009; Stutzbach et al., 2013). One carrier (III:18) from family 1 had severe neuronal loss in the CA regions and subiculum. Overall, the staining of pelf2 α was more prominent than pPERK (Fig. 2A and D). All elderly nondemented controls showed a low to moderate degree of pPERK staining in the hippocampus. *EIF2AK3* carriers had significantly more positive staining than nondemented controls in the hippocampus ($p = 0.04$) and temporal region ($p = 0.03$). For pelf2 α , a trend for more positive staining was only observed in the hippocampus of *EIF2AK3* carriers compared with nondemented controls ($p = 0.07$). We found no difference in all examined regions when comparing *EIF2AK3* carriers with AD non-*EIF2AK3* carriers; all *EIF2AK3* carriers had Braak stage 6 with extensive tau pathology in the hippocampus, frontal, temporal, and parietal cortices.

We used Western blot analysis with a series of buffers with increasing strength to solubilize proteins to investigate biochemical alteration of pPERK. One band of approximately 140 kDa in low salt, representing pPERK, was found in both *EIF2AK3* mutation carriers and AD cases. We found no differences in banding and solubility of pPERK between carriers of *EIF2AK3* and AD non-*EIF2AK3* carriers (Supplementary Fig. 2).

4. Discussion

This is the first study to investigate the role of rare variants in *EIF2AK3* in patients with AD. We performed WES in 8 Dutch families

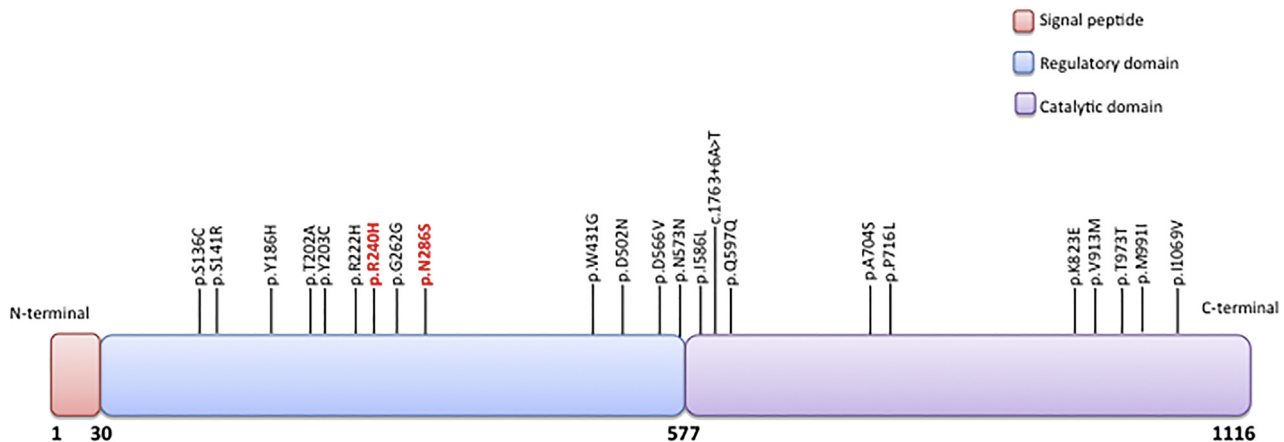


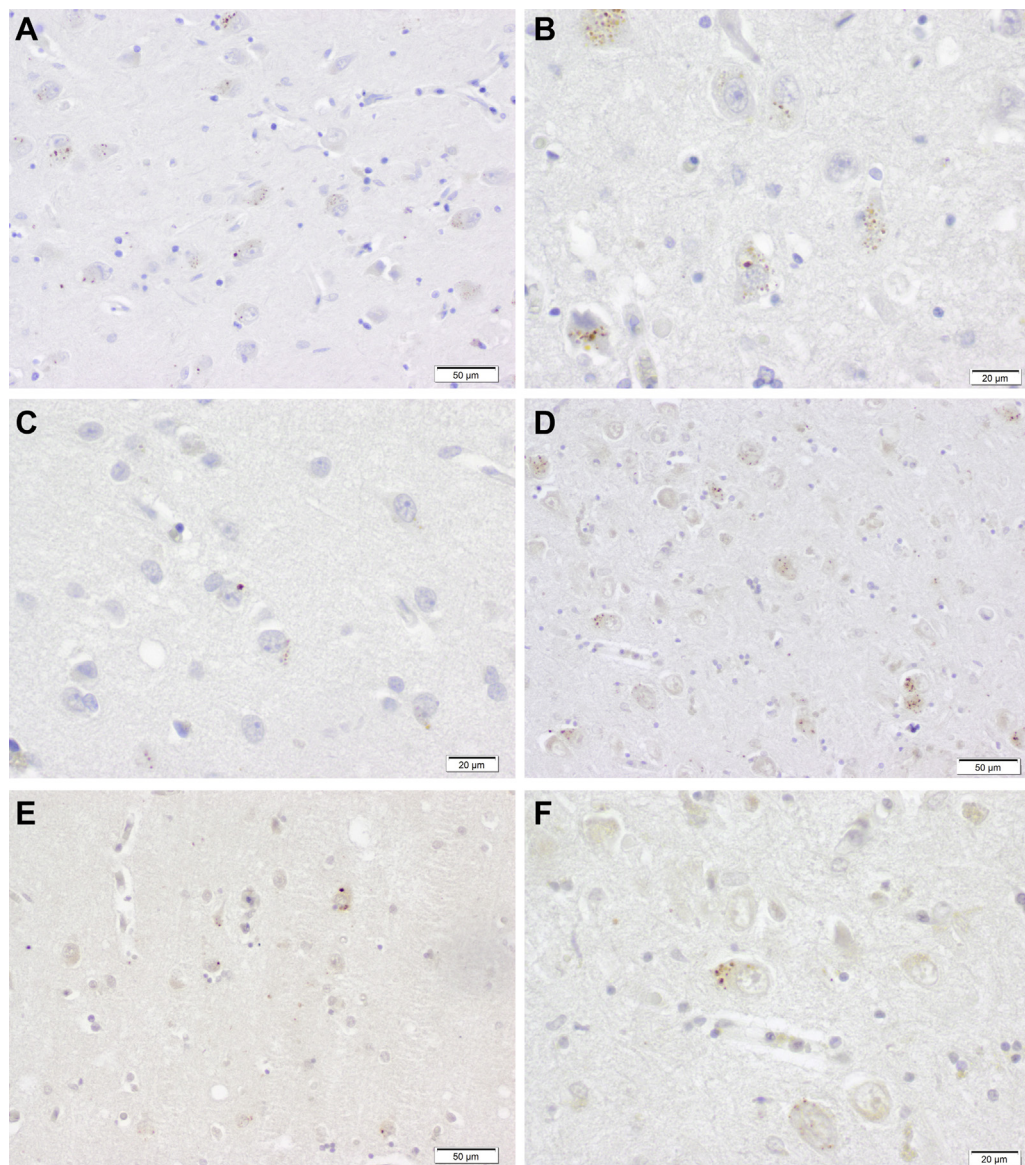
Fig. 1. Schematic representation of *EIF2AK3* gene and relative position of the *EIF2AK3* variants found in the present study. The gene *EIF2AK3* contains 1116 amino acids and is composed of a signal peptide, a regulatory domain, and a catalytic domain. Variants highlighted in red are found in the family-based analysis. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2Scoring of inclusions for pelf2 α and pPERK antibodies

ID	Braak stage	Age at death	PMD	pelf2 α			pPERK		
				Frontal	Temporal	Hippocampus	Frontal	Temporal	Hippocampus
Carrier III:15 (R240H)	6	83	5:30	–	++	++++	–	+	+++
Carrier III:18 (R240H)	6	91	4:20	+	++	+++	+	+	++++
Carrier III:7 (N286S)	6	70	6:20	+	+++	++++	+	++	++++
AD non-EIF2AK3 carrier 1	5	95	7:00	+	++	++++	–	+	+++
AD non-EIF2AK3 carrier 2	5	62	4:40	+	+++	++++	–	+	+++
AD non-EIF2AK3 carrier 3	5	71	5:50	+	+	++++	–	–	+++
ND control 1	4	96	4:10	–	++	+++	–	–	++
ND control 2	2	80	4:25	–	++	++	–	–	++
ND control 3	2	90	5:45	–	+	++	–	–	+

Semiquantitative scoring of inclusions for pelf2 α and pPERK for carriers with *EIF2AK3* variants, AD non-*EIF2AK3* controls and ND controls.

Key: –, negative; +, rare; ++, low density (up to 10%); +++, moderate density (11%–30%); +++++, high density (>30%); AD, Alzheimer's disease; ND, nondemented; PMD, postmortem delay.

**Fig. 2.** Immunohistochemical staining of pPERK and pelf2 α in the AD cases with *EIF2AK3* mutations. Activated pPERK and pelf2 α was found in the hippocampus and temporal regions (A–F). High numbers of pPERK stained cells were observed in the cornu ammonis (A) and subiculum (B) of the hippocampus, and lesser numbers were found in the temporal regions (C–F).

with a high burden of AD and identified *EIF2AK3* as a candidate gene in 2 families. Subsequently, gene-based analysis in an independent Dutch WES cohort showed suggestive association of *EIF2AK3* with AD. These effects seemed to be mainly driven by variant p.R240H. Although pPERK and pelf2 α staining was more prominent in *EIF2AK3* carriers than in controls, it was similar to AD non-*EIF2AK3* carriers.

We identified 2 distinct variants in *EIF2AK3* segregating with AD in 2 different families, although unaffected carriers found in each family suggested incomplete penetrance; however, they may still develop AD at an older age. The association of an *EIF2AK3* variant with AD has been reported previously, wherein 1 SNP (rs7571971) in *EIF2AK3* was associated with AD in *APOE* ϵ 4 carriers, but not independent of *APOE* (Liu et al., 2013); however, to date, no studies have examined the association of rare variants in *EIF2AK3* with the risk of AD. The gene burden test of *EIF2AK3* in our Dutch AD exome data set supported this association of rare variants with AD ($p=0.03$), in which it was mainly driven by the variant p.R240H with a CADD score of 31, but we were unable to confirm the association between p.R240H and AD in an additional cohort from the Rotterdam Study, although there was a trend toward association with AD. A possible explanation for the lack of significance is the relatively small sample size for this rare variant. Notably, the high frequency of *APOE* ϵ 4 carriers among the *EIF2AK3* carriers in the 2 families and in the Dutch AD exome data set further support an association of *EIF2AK3* variants with AD in *APOE* ϵ 4 carriers as indicated by Liu et al. (Liu et al., 2013), although similar results were found for the association tests with and without *APOE* as covariate. Studies with larger sample sizes are needed to examine the effects of rare variants in *EIF2AK3* on the risk of developing AD.

The potential significance of *EIF2AK3* variants in our families also lies in the fact that PERK is a transmembrane protein involved in learning, memory, and UPR (Devi and Ohno, 2014; Rozpedek et al., 2015). Our hypothesis was that variants in *EIF2AK3* may enhance PERK signaling, resulting in increased phosphorylation of tau by glycogen synthase kinase 3 β (GSK3 β) and amyloidogenesis (by beta-secretase 1 [BACE1]). Previous studies have indicated that PERK-eIF2 α signaling is involved in the modulating of tau phosphorylation and APP processing in AD (Devi and Ohno, 2014; Hoozemans et al., 2009; Nijholt et al., 2013), but that it is also correlated with the level of tau pathology in progressive supranuclear palsy and AD (Hoozemans et al., 2009; Stutzbach et al., 2013). pPERK immunoreactivity also colocalized with GSK3 β in neuronal cells, which is involved in tau phosphorylation (Hoozemans et al., 2009; Nijholt et al., 2013). Treatment with a PERK inhibitor (GSK2606414) in transgenic mice with frontotemporal lobar degeneration and overexpression of p.P301L mutation resulted in reduced GSK3 β levels and tau phosphorylation compared with transgenic mice without PERK inhibitor treatment (Radford et al., 2015). Moreover, *PSEN1* (5XFAD)-mutated mice with PERK haploinsufficiency had lower levels of BACE1 than those with normal PERK levels, resulting in lower amyloid-beta peptide levels and plaque burden, as well as fewer memory deficits and cholinergic neurodegeneration (Devi and Ohno, 2014). Reduced synaptic plasticity and spatial memory deficits were found in the APP/PS1 AD mice model with PERK haploinsufficiency (Ma et al., 2013). Although these studies supported a role of PERK signaling in the pathogenesis of AD, functional experiments are needed to confirm the effect of *EIF2AK3* variants.

The increase of PERK-eIF2 α signaling in the *EIF2AK3* carriers is supported by the more positive staining of pPERK and pelf2 α compared with nondemented controls, indicating an increased activation of UPR. This increased UPR has also been observed in AD and progressive supranuclear palsy patients in previous studies (Hoozemans et al., 2005; Stutzbach et al., 2013). However, we did

not find any differences in pPERK and pelf2 α staining between *EIF2AK3* carriers and AD non-*EIF2AK3* carriers, suggesting *EIF2AK3* mutation carriers might not induce more UPR activation than other AD patients. A possible explanation is that *EIF2AK3* mutation carriers may trigger UPR activation early in the disease process, without the ability to observe this at the end-stage AD.

The main limitation of our study is the family-based analysis used to identify the candidate genes; we only selected genes containing rare variants in at least 2 families for follow-up. We cannot rule out the possibility that other possible candidates in the families were missed. However, this method has previously been successfully used by Cruchaga et al., resulting in the identification of the genetic association of *PLD3* with AD (Cruchaga et al., 2014). Furthermore, *EIF2AK3* was the only gene in our candidate list involved in the pathogenesis of AD. Another limitation is the limited available samples of related cases and (old) nondemented controls in some families to analyze segregation; some nondemented controls may still develop dementia at older age. Finally, the frequency of *APOE* ϵ 4 is high in some families, and *APOE* ϵ 4 segregates with the disease in some of them. This is also true for family 4, in which variant p.N285S was found; 4 patients and 1 individual with memory complaints carried at least 1 copy of *APOE* ϵ 4. However, all 4 patients carrying p.N285S and *APOE* ϵ 4 had early-onset AD, indicating a possible additional effect of genetic variation in *EIF2AK3* on the risk of AD among *APOE* ϵ 4 carriers, as indicated in a previous study (Liu et al., 2013). Future analyses in larger case-control studies are necessary to confirm this association.

In conclusion, our study showed that rare variants in *EIF2AK3* may be associated with an increased risk of AD based on segregation among the patients with AD in 2 families and a gene-based analysis in the Dutch WES cohort. Immunohistochemistry confirmed more activation of UPR, characterized by increased pPERK and pelf2 α in AD patients compared with nondemented controls, but not between *EIF2AK3* carriers and AD noncarriers. Further studies are needed to investigate the full contribution of rare variants in *EIF2AK3* in the development of AD.

Disclosure statement

The authors declare no actual or potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.neurobiolaging.2018.08.016>.

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