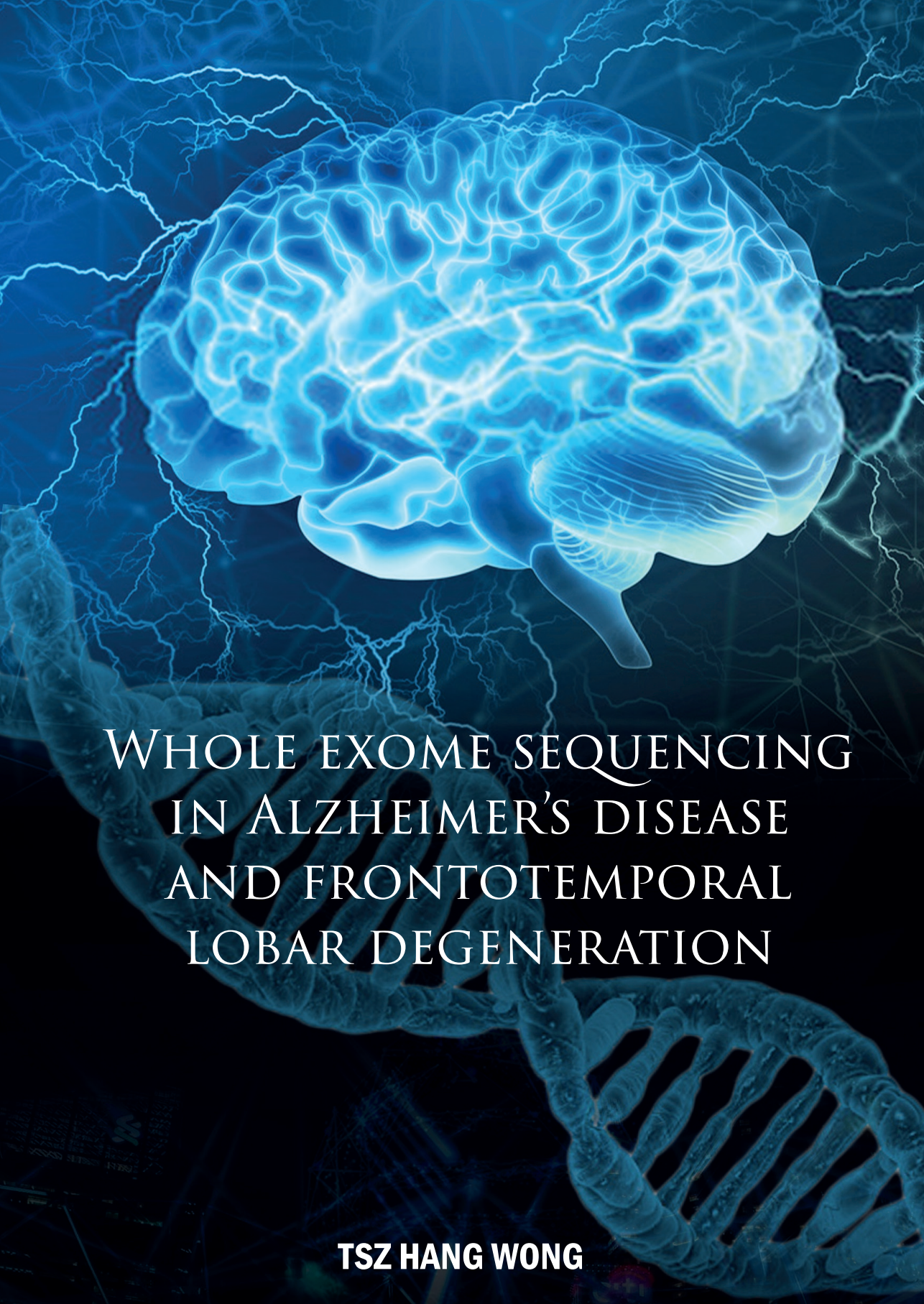

Whole exome sequencing in Alzheimer's disease and frontotemporal lobar degeneration

TSZ HANG WONG

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en frontotemporale lobaire degeneratie

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CHAPTER 1

1

General introduction and scope of the thesis

Dementia is a disorder characterized by cognitive impairments and/or behavioral disturbances that interfere with the ability of daily functioning.[1] Alzheimer's disease (AD) is the most common cause of dementia, characterized by progressive memory loss and other cognitive impairment including language, executive functions and visuospatial skills.[2] In contrary, frontotemporal dementia (FTD) is the second most common cause of dementia before the age of 65 years, predominantly characterized by behavioral disturbances and/or language deficits.[3] Genetic factors are involved in both AD and FTD, with a high heritability up to 50% in FTD.[4] High penetrant mutations in presenilin 1 (*PSEN1*), presenilin 2 (*PSEN2*) and amyloid precursor protein (APP) are major genetic causes of autosomal dominant early-onset AD (EOAD).[5] Mutations in microtubule associated protein tau (*MAPT*), progranulin (*GRN*) and hexanucleotide repeat expansion within the non-coding region of the chromosome 9 open reading frame 72 (*C9orf72*) are responsible for the majority of FTD cases.[6] Although the majority of familial AD and FTD cases have been explained by these genetic mutations, there still exists familial cases with unidentified mutations. Currently, no cure is available for both diseases. Studying genetic factors provide us knowledge about the disease mechanism, which is essential for the development of new therapeutic strategies.

During the last decade, genetics in AD and FTD have made major steps, predominantly by introducing genome-wide association studies (GWAS) and next-generation sequencing (NGS) studies in the genetic research, explaining a subset of the missing heritability.[6, 7] In contrary to GWAS studying common risk factors with a small effect contributing to the development of disease, NGS has enabled us to investigate the effect of rare variants with larger effect size.[8] Whole exome sequencing (WES), a NGS technique focusing on protein-coding regions of the genome, is a cost-effective approach to identify mutations with probable damaging effect on the protein function.

In this chapter we review the genetics forms in AD and FTD with their corresponding clinical and pathological features.

Whole exome sequencing (WES)

NGS using parallel sequencing approach to sequence exomes, specific loci or genomes, has enabled us to investigate the involvement of rare variants in distinct disease traits.[8] WES, a high throughput sequencing method sequencing protein-coding regions, may identify the underlying genetic defect, particularly in small families or single patients in which traditional linkage analysis is troublesome. In the last decade, WES has successfully identified novel mutations in AD and FTD.[6, 9] However, WES has some limitations: 1. A high error rate due to sequencing errors or incorrect base calling compared to traditional Sanger sequencing. 2. Some genetic variants, such as copy number variants, variants in non-coding sequences or repeat expansions cannot be

detected. 3. Rare variant analysis is challenging due to low statistical power due to minor allele frequencies, population stratification and false positive findings.[7] For the latter issue, burden tests that compare the cumulative frequency by collapsing rare variants in a single gene or a specific genomic region could partly solve power issues, although large sample sizes are still needed to detect signals which sustain multiple testing.[10]

Alzheimer's disease

AD is clinically characterized by progressive memory loss and other cognitive impairment including language, executive functions and visuospatial skills.[1] Memory impairments is the most common initial clinical presentation of AD, but atypical presentation including behavioral changes, language and dysexecutive problems are also observed, and varied from 6-14% of AD cases.[2] Neuropathologically, depositions of extracellular amyloid plaques and intracellular neurofibrillary tangles are the pathological hallmarks of the disease.[11] AD is subdivided into EOAD and late-onset AD (LOAD) using a cut-off age of 65 years. EOAD accounts for about 1-2% of AD cases, and in around 13% of these cases an autosomal dominant pattern of inheritance is found.[5] Although a subset of AD families with an autosomal dominant inheritance has been explained by single gene mutation (also referred as Mendelian forms), the majority of AD cases are genetically complex involving an interaction between genetic and environmental factors.[12] An overview of gene defects associated with AD is presented in Table 1.

Table 1. Genes associated with Alzheimer's disease

Gene	Gene locus	Inheritance	EOAD/LOAD	Type of mutation	Implicated disease pathway
PSEN1	14q24.2	AD	EOAD	missense	APP processing
PSEN2	1q42.13	AD	EOAD	missense	APP processing
APP	21q21.3	AD	EOAD	missense, copy number variation	APP processing
APOE	19q13.32	Risk factor	LOAD	missense	Lipid metabolism
TREM2	6p21.1	Risk factor	LOAD	missense	Immune response
PLD3	19q13.2	Risk factor	LOAD	missense	Lipid metabolism, immune response
SORL1	11q24.1	AD or risk factor	EOAD/LOAD	loss of function and missense	Endocytosis, lipid metabolism
ABCA7	19p13.3	AD or risk factor	EOAD/LOAD	loss of function and missense	Lipid metabolism
UNC5C	4q22.3	Risk factor	LOAD	missense	Neuronal development
AKAP9	7q21.2	Risk factor	LOAD	missense	Signal transduction

AD, autosomal dominant; EOAD, early-onset Alzheimer's disease; LOAD, late-onset Alzheimer's disease.

Mendelian forms in AD

Highly penetrant mutations in *PSEN1*, *PSEN2* and *APP* with an autosomal dominant pattern of inheritance explain for approximately 5-10% of EOAD patients,[5, 13-15] and were rarely found in LOAD patients.[16] To date, more than 280 mutations have been found in *PSEN1*, *PSEN2* and *APP* genes (www.molgen.ua.ac.be/ADMutations).[17]

APP

The *APP* gene is located at chromosome 21, and produces different transcripts by alternative splicing.[18] The protein APP is cleaved into fragments via non-pathogenic pathway (by α and γ-secretases) and amyloidogenic pathway (by β- and γ-secretases).

Missense and copy number mutations in *APP* have been reported, and account for less than 1% of the EOAD cases.[5, 17] The majority of the *APP* mutations are located at the γ-secretases cleavages sites or on exons 16 and 17. However, recessive mutations (A673V and E693Δ) have also been reported in families with AD.[19, 20] The clinical phenotypes of *APP* mutations carriers include AD and/or cerebral amyloid angiopathy (CAA),[21] and the age at onset varies from 32-64 years.[22] Neuropathologically, increased amyloid beta 40 (Aβ40) deposits in the cerebral vessels consistent with diagnosis of CAA have been observed.[21]

PSEN1 and PSEN2

PSEN1 is located at chromosome 14, and its homologue *PSEN2* is located at chromosome 1. Both genes encode for integral membrane proteins that contain nine transmembrane domains with a hydrophilic intracellular loop region.[23] *PSEN1* and *PSEN2* are both key components of γ-secretases, which processes APP by cleaving into amyloid beta (Aβ) fragments.18 Mutations in these genes impair the proteolytic activity of γ-secretases, resulting in elevated amyloid-beta 42 (Aβ42) and a higher Aβ42/Aβ40-ratio.

Mutations in *PSEN1* are the most common cause of EOAD accounting for 6% of the cases.[5] More than 200 mutations in *PSEN1* have been reported including missense, insertions and deletions.[17] In contrary, only 16 pathogenic *PSEN2* mutations have been identified so far, and accounts for approximately 1% of EOAD.[5] Mutations in *PSEN1* and *PSEN2* are highly penetrant, although risk factors and nonpathogenic variants are also reported in these genes.[24] Variable age at onset among the mutation carriers ranging from 23 to 71 years had been reported, even within families with the same mutations.[22] Overall, younger age at onset has been reported for *PSEN1* carriers (with mean 43 years) than for *PSEN2* carriers (with mean 58 years). Clinical heterogeneity is frequently reported including initial memory impairment, behavioral problems and language impairment.[25, 26] In *PSEN1* carriers, atypical cognitive presentations and pyramidal signs are more frequently observed in mutations beyond codon 200, while mutations before codon 200 were more frequently associated with younger age at onset.[26]

Neuropathologically, *PSEN1* and *PSEN2* mutation carriers often have greater amount of neocortical senile plaques and higher Aβ42/Aβ40 ratio than sporadic AD cases.[27] Furthermore, cotton wool plaques, which are large amyloid aggregates lacking the distinct amyloid core and prominent dystrophic neurites, are more frequently seen in *PSEN1* mutations carriers with spastic paraparesis than sporadic cases.[27, 28]

Genetically complex forms

APOE

Apolipoprotein E (*APOE*) gene is located at chromosome 19, and contains three isoform that differ at amino acid residues 112 and 158: *APOE* ε2, *APOE* ε3 and *APOE* ε4.[23] *APOE* ε4 is associated with an increased risk for developing LOAD compared to individuals with the most common genotype *APOE* ε3, with three-fold increased risk of AD for individuals carrying one ε4 alleles to ten-fold increased risk for those with two ε4 alleles.[18] Furthermore, *APOE* ε4 also increased risk in EOAD patients who carry at least one copy of ε4, and in particularly who have a positive family history.[5] Although *APOE* ε4 is associated with an increased risk to develop AD, carrying this allele is neither necessary nor sufficient to cause AD. Up to 75% of the people who carry one allele of *APOE* ε4 did not develop AD.[29] In contrary, *APOE* ε2 has a protective effect against AD by lowering the risk to 0.6 times in homozygous state compared to the common genotype.[30]

Various studies have replicated the relationship between *APOE* and AD including its clinical and pathological correlation.[31, 32] The number of *APOE* ε4 alleles is associated with an earlier age at onset, and an increased rate of cognitive and functional decline. Furthermore, *APOE* ε4 carriers with AD showed a higher rate of atrophy of the entorhinal cortex and hippocampus than *APOE* ε4 non-carriers with AD. Pathologically, more (neuritic) senile plaques have been found in the brains of AD cases carrying at least one copy of *APOE* ε4 than non-carriers, and this number is even higher among AD cases carrying two copies of *APOE* ε4.[32]

SORL1

The gene Sortilin-related receptor 1 (*SORL1*) encodes for sorting-related receptor with A-type repeats, and is involved in neuronal sorting process including intracellular transport, which is important for APP processing and the generation of AB peptides.[33]

Genetic association of *SORL1* variants with AD was initially reported as a risk factor for LOAD in a case-control study,[34] and this increased risk has been further replicated in other GWAS and meta-analysis.[35-38] In the WES era, rare coding variants in *SORL1* have been found to be enriched in AD cases compared to controls, and in particularly, rare protein truncating variants (PTV) have been exclusively found in AD cases.[39-41]

Although rare coding missense variants in *SORL1* have been reported in patients with EOAD as well as LOAD, little is known about the damaging effects and the disease penetrance of the reported variants. Only a few studies reported co-segregation of rare variants in *SORL1* in small families.[41, 42] Two possible pathomechanisms of *SORL1* mutants have been hypothesized, possibly depending on mutation type: 1) impaired sorting of full-length APP into the retromer-recycling endosome pathway; 2) failure to slow trafficking of APP to cell surface.[33, 43]

The clinical presentation of *SORL1* carriers included classical phenotype of AD with memory impairment, although early neuropsychiatric and parkinsonian features have also been reported.[42, 44]

Rare variants associated with AD risk

Large collaborative efforts in GWAS has successfully identified multiple genetic loci, associated with increased risk of AD.[45-47] However, these genetic loci, usually containing several genes, have only a small effect on AD risk with odd ratios < 2, and emerging evidence suggested the existence of rare variants with larger effect size which is associated with AD risk. A detailed list of the identified genetic loci can be found at www.Alzgene.org.

Since the implementation of next generation sequencing, multiple rare variants with larger effect size associated with AD have been found.[48] Triggering Receptor Expressed on Myeloid Cells 2 (*TREM2*) variants have been implicated to increase AD risk in two independent studies,[49, 50] and the variant p.Arg47His has been replicated in many studies.[38, 51-54] TREM2 is highly expressed by microglial cells in the brains,[49, 55] and is involved in the regulation of phagocytosis, inhibition of inflammatory signaling, cytokine production and secretion in microglia.[56] Evidence indicated that mutation in *TREM2* could result in an impaired clearance of Aβ and microglia activation.[57]

Loss of function variants in ATP Binding Cassette Subfamily A Member 7 (*ABCA7*), a gene initially identified in GWAS studies,[45] was discovered to be associated with increased AD risk in Icelandic population.[58] Sequential analysis in several case-control studies has confirmed an enrichment of loss of function variants in AD patients comparing with controls.[59-62]

Rare coding variants in Phospholipase D3 (*PLD3*) has been implicated to increase the risk of LOAD by demonstrating of cosegregation of *PLD3* in two large AD families using WES followed by genetic association in large case-controls series.[63] However, this genetic association could not be replicated with either EOAD or LOAD in most of the studies.[64-68] Sequencing studies have also linked rare variants in genes like *UNC5C* and *AKAP9* with risk of AD, but these association is uncertain due to limited replication studies and functional experiment.[69, 70]

Frontotemporal dementia

FTD is the second most common presenile form of dementia, and is characterized by progressive behavioral changes, executive deficits and/or language impairment. Arnold Pick has reported the first patient with FTD in 1892, who described a patient with progressive aphasia, dementia and lobar atrophy.[71] In 1911, Alois Alzheimer referred the neuropathological features as Pick bodies and named the clinicopathological entity as Pick Disease.[72]

The prevalence of FTD ranges from 1 to 26 per 100,000 inhabitants with age of 65 or younger,[73-76] and a frequency of 2.7 per 100,000 inhabitants has been reported in the Netherlands.[77] The average age at onset is around 50-60 years, and higher age at onset of over 70 years has been reported in 10% of the FTD cases.[4]

Clinical features

FTD is clinically divided into three subtypes: behavioural variant, progressive non-fluent aphasia (PNFA) (also known as non-fluent variant PPA) and semantic dementia (SD) (also known as semantic variant PPA).[4] The latter two and together with logopenic aphasia are classified as primary progressive aphasia (PPA). Behavioral variant FTD (bvFTD) is characterized by early behavioural changes and impairment in executive functions. Patients with PNFA present with slow, labored and halting speech accompanied with agrammatism.[3] In contrast, patients with SD usually have an impaired word finding difficulties and word comprehension but a fluent speech. Motor neuron disease (MND) can occur in conjunction with FTD in about 10% of all cases, and is more often observed in bvFTD, and rarely in PPA. Furthermore, early parkinsonian symptoms including corticobasal syndrome (CBS) and progressive supranuclear palsy (PSP) like symptoms has been found in up to 20% of patients with FTD.

Neuropathology

The term frontotemporal lobar degeneration (FTLD) encompasses the pathological entity of clinical FTD subtypes, characterized by atrophy of predominantly the frontal and temporal lobes.[78] The pathology of FTLD is heterogeneous, and can be classified into distinct subtypes based on the aggregation of intracellular or intranuclear disease-specific protein (also referred as inclusions).[78, 79] Based on these inclusion and molecular defects, FTLD can be classified into four main subtypes: FTLD with tau (FTLD-Tau) in ~40 % of the cases, transactive response DNA-binding protein of 43 kDa (FTLD-TDP) in ~50% of the cases, FET protein (including Fused in Sarcoma (FUS), Ewing Sarcoma (EWS) and TATA binding associated factor 15 (TAF-15)) protein aggregation (~5-10%) and FTLD-ubiquitin proteasome system (UPS) (<1%).[80, 81]

The main neuropathological finding of FTLD-tau is aggregation of hyperphosphorylated tau (ptau) protein in the neuronal and glial cells,[80] also called as tauopathy, produced by alternative splicing of exon 2, 3 and 10 of the microtubule associated tau (*MAPT*) gene, and accounts for ~40% of all FTLD cases.

FTLD-TDP-43 has been suggested as the most common FTLD-type representing approximately 50% of the FTLD cases.[80, 82] The hallmarks of FTLD-TDP are neuronal cytoplasmic inclusions (NCI) and dystrophic neurites (DN) which are immunoreactive (IR) for TDP-43, ubiquitin and p62. Four different FTLD-TDP subtypes (A-D) has been proposed based on the morphology and distribution of these TDP-43 IR aggregates: Type A is characterized by abundance of short DN, compact NCI and lentiform neuronal intranuclear inclusions (NII), predominantly in the second layer of the neocortex; Type B represents cases with diffuse granular NCI and a few DN which are distributed in all layers; Type C cases show long thick DN with a few NCI in all layers; Type D cases have abundant lentiform NII and short DN in the neocortex, but only rare NCI. Although the majority of the FTLD-TDP cases could be classified into one of these TDP subtypes, a combination of different FTLD-TDP subtypes has been observed in up to 19% of the FTLD-TDP cases.[80,81] Additionally, a small number of cases has been found characterized by granulofilamentous neuronal inclusions, abundance of grains and oligodendroglial inclusions referred to as TDP type E.[83]

The remaining FTLD cases (~10%) are subdivided into FTLD-FET or FTLD-UPS, and are characterized by tau- and TDP43-negative, but ubiquitin positive inclusions.

Genetics of FTLD

A positive family history, defined as at least one affected first-degree family member with dementia, ALS or Parkinson's disease, has been reported in 30-50% of patients with FTD.[4] A positive family history has been more commonly observed in bvFTD cases than SD and PNFA. An autosomal dominant mode of inheritance has been reported between 10-27% of patients with FTD. A mutation in one of the three genes are the major genetic causes in FTD, inherited in autosomal dominant mode: *MAPT*,[84] *GRN*[85, 86] and *C9orf72*.[87, 88] Table 2 lists the causative genes reported in FTLD.

MAPT

The *MAPT* gene is involved in microtubules stabilization, and was identified as the first genetic cause for familial FTD.[84] Two possible mechanisms has been suggested for pathological deposits of ptau in *MAPT* mutations: 1) some mutations disrupt the binding of tau protein to microtubules and thereby reduce microtubule assembly; 2) other mutations affect the splicing regulation of tau protein resulting in an imbalance of 3R:4R tau isoform ratios.[89] Over 44 different pathogenic *MAPT* mutations have been reported (http://www.molgen.ua.ac.be/FTDmutations), predominantly located

Table 2. Genes associated with frontotemporal dementia

Gene	Gene locus	Inheritance	Phenotype	Pathology	Implicated disease pathway
MAPT	17q21.1	AD	FTD, PSP, CBS	Tau	Toxic aggregation (defect in neuronal cytoskeleton)
GRN	17q21.32	AD	FTD, CBS	TDP type A	Autophagy, Lysosomal pathway, inflammation
C9orf72	9q21.2	AD	FTD and/or ALS	TDP type A and/or B	Toxic RNA or repeat dipeptides aggregation
CHMP2B	3p11.2	AD	FTD	UPS	Autophagy, Lysosomal pathway
TARDBP	1p36.22	AD	FTD and/or ALS	TDP unspecified	DNA/RNA metabolism
VCP	9p13.3	AD	IBMPFD, FTD and/or ALS	TDP type D	Autophagy
SQSTM1	5q35	AD	FTD and/or ALS	TDP type B	Autophagy
hnRNPA1/ hnRNPA2B1	12q13.1/ 7p15	AD	FTD and/or ALS	unspecified	RNA metabolism; direct interaction with TDP-43
CHCHD10	22q11.23	AD	FTD and/or ALS	unspecified	Mitochondrial dysfunction, synaptic integrity
TBK1	12q14.2	AD	FTD and/or ALS	TDP type A or B	Autophagy, inflammation
OPTN	10p13	AR	FTD and/or ALS	TDP type A	Autophagy
UBQLN2	Xp11.21	AD	FTD and/or ALS	unspecified	Autophagy
FUS	16p11.2	AD	ALS (and FTD)	FUS	DNA/RNA metabolism
TMEM106B	7p21.3	Risk factor	FTD	NA	Regulation of lysosomal function and progranulin pathways

AD, autosomal dominant; ALS, amyotrophic lateral sclerosis; AR, autosomal recessive; CBS, corticobasal syndrome; FTD, frontotemporal dementia; FUS, fused in sarcoma; IBMPFD, inclusion body myositis with early-onset Paget disease and frontotemporal dementia; NA, not available; TDP, Tar DNA-binding protein; UPS, ubiquitin proteasome system.

between exon 9 and exon 13.[17, 90] The frequency of *MAPT* mutations varied from 5% to 20% in familial cases depending on the geographical distribution.[6, 91-93]

The clinical presentation of *MAPT* mutations carriers is heterogeneous, with bvFTD as the most common phenotype, and less commonly memory impairment, semantic deficits and extrapyramidal symptoms.[6] Some *MAPT* mutations carriers presented with prominent atypical parkinsonism resembling PSP and CBS, such as p.S303S and p.S305S mutations.[90] The penetrance of the mutations is high, but unaffected mutation carriers have been reported.[94, 95] The age at onset varied from 45 to 65 years.[88]

The pathological features of *MAPT* mutations are neuronal loss and gliosis accompanying with neuronal inclusions of ptau protein in cortical and subcortical gray and white matter.[80] The pathological diagnosis of *MAPT* mutations includes Pick disease, PSP, CBD and GGT.[90] In general, mutations causing a relative increase of 4R tau isoform by alternate splicing of exon 10 are associated with neuronal and glial p-tau inclusions resembling the pathology of sporadic PSP and CBD, whereas mutations outside this splicing region are associated with Pick bodies containing predominantly 3R or NFT containing both 3R and 4R tau isoforms.[80]

GRN

Progranulin (PGRN) is a growth factor that is involved in various processes including wound healing, cell proliferation, tumor growth, neuroinflammation, neuronal survival and neurite outgrowth.[96] *GRN* mutation was identified as the second gene which can cause FTD.[85, 86] Pathogenic mutations in *GRN* resulted in null alleles, leading to reduced function of progranulin (haploinsufficiency). To date, over 70 loss of function *GRN* mutations have been found, representing 5-20% of familial FTD and 1-5% of sporadic FTD cases.[17, 97] The majority of pathogenic mutations are protein-truncating including nonsense, splice-site and frameshift mutations, but partial deletions and a complete deletion of *GRN* have also been described.[98] Additionally, several missense variants in *GRN* have been reported, however, the pathogenicity of many of these variants are unclear except for p.A9D.[6] Although the exact pathomechanism how *GRN* mutations cause FTD is unknown, accumulating evidence suggested that PGRN deficiency results in lysosomal dysfunction.[96]

Patients with *GRN* mutations have a highly heterogeneous clinical phenotype, with bvFTD as the most common phenotype followed by PNFA.[97] Hallucinations and delusions have been frequently reported with a frequency up to 25%.[99, 100] Clinical signs of MND are rarely reported.[101] Furthermore, extrapyramidal signs fulfilling the diagnosis of CBS have also been observed among *GRN* mutation carriers.[100, 102, 103] *GRN* carriers had a wide range of age at onset, ranging from 35 to 89 years.[92, 100, 102] The penetrance of mutation carriers is estimated to be 90% at age of 70,[97] and the median disease duration is 7.0 years.[101]

The neuropathology of *GRN* carriers is characterized by many DN accompanied with crescentic and oval NCI, most abundant in layer two of the neocortex, consistent with FTLD-TDP type A.[80] Furthermore, a moderate number of lentiform NII are present. Also, DN, NCI and NII are frequently found in the striatum, and variable numbers of NCI are found in the dentate gyrus, most of them with a granular morphology.

C9orf72

In 2011, a pathogenic GGGGCC (G_4C_2) hexanucleotide repeat expansion in the non-coding region of *C9orf72* has been identified as the most common genetic cause for FTD and/or amyotrophic lateral sclerosis (ALS),[87, 88] explaining 21% of familial FTD and 6% for sporadic FTD in North American and European populations.[104, 105] Higher frequency has been reported in ALS cohorts with an average of 37% for familial cases and 5% for sporadic cases.[105] *C9orf72* repeats expansion has an autosomal dominant inheritance mode, and anticipation in the family has rarely been reported.[106] The minimal repeat size associated with FTD and/or ALS is not fully clear, but the cut-off is usually set on 30. Variable repeat size varying from 2-20 repeats in healthy individuals to a larger repeat size of a few hundred to several thousands in patients with FTD and/or ALS has frequently been observed.[107, 108] Furthermore, tissue-specific variation in repeat size with a large repeat lengths in brain tissue but shorter in blood, has also been found, indicating somatic mosaicism.[109]

The underlying disease mechanism of *C9orf72* repeats expansion causing FTD and ALS is not fully known. Three possible disease mechanism have been suggested: 1) loss of function, 2) gain of function through RNA toxicity, and (3) toxicity of dipeptide repeat proteins (DPRs) translated from unconventional repeat- associated non-ATG (RAN) translation of G_4C_2 repeats.[110]

Clinically, *C9orf72* expansions carriers also had a wide range in age at onset from 27 to 83 years, and the disease duration ranged from 1 to 22 years.[105, 108] BvFTD, ALS or combination of FTD-ALS are the most common clinical presentation of mutation carriers, and less frequently semantic dementia and non-fluent variant.[108, 111] Furthermore, initial amnestic symptoms presenting with prominent memory impairment may occur, and may fulfill with the clinical diagnosis of AD.[1, 108] Psychotic symptoms including hallucinations and/or delusions have commonly been reported in *C9orf72* expansion carriers compared to non-carriers,[112, 113] and could be misdiagnosed with psychiatric disorders such as schizophrenia or bipolar disorders.[114]

Neuropathologically, FTLD-TDP type B is linked to *C9orf72* expansions carriers.[80] However, a combination of TDP type A and type B, characterized by moderate to numerous NCI in deeper cortical layers with a high proportion of granular NCI, and occasionally accompanied with abundant threads and dots, has been found in subsets of *C9orf72* expansions carriers.[81] Furthermore, six different forms of neuronal inclusions containing DPR proteins, produced from RAN translation of the G_4C_2 repeats, have also been observed in the brain tissue of *C9orf72* expansions carriers.[115, 116]

Rare genetic causes of FTLD

CHMP2B

The first splice site mutation in Charged multivesicular body protein 2B (*CHMP2B*) was identified in a large Danish autosomal dominant FTD family with linkage to chromosome 3.[117] This gene encodes for a component of the Endosomal Sorting Complex Required for Transport III, which is involved in protein degradation through endosome-lysosome pathway and autophagy.[118] The contribution of *CHMP2B* mutations to FTD is small, representing less than 1%.

Clinically, bvFTD is the common phenotype of *CHMP2B* mutations carriers.[119] Extrapyramidal symptoms might occur in the advanced stage of the disease process, and ALS has occasionally been reported.[120, 121]

Neuropathologically, abundant NCI that are ubiquitin-IR, but negative for TDP-43 and FUS, has been found in dentate granular layer of the hippocampus and the adjacent neocortex.[122, 123] This pathology is consistent with FTLD-UPS.[80]

TARDBP

TAR DNA binding protein (*TARDBP*) encodes for TDP-43, and is involved in the regulation of transcriptional activity of messenger RNA splicing, exon skipping and microRNA biogenesis.[124] In FTLD and/or ALS, TDP-43 is a major component of ubiquitin positive, but tau negative inclusions.[82] Mutations in *TARDBP* was initially reported as causative gene for ALS representing approximately 3% of ALS cases,[125] but other studies have reported association with FTD with a mutation frequency close to 1%.[126-129] Most pathogenic *TARDBP* mutations are clustered in exon 6, in the conserved C-terminal glycine-rich domain.[125, 127]

The clinical presentation includes bvFTD and/or ALS, and initial language impairment fulfilling the clinical diagnosis SD or extrapyramidal signs have also been reported.[128, 130, 131] Only a few studies reported neuropathological findings of *TARDBP* mutation carriers, showing a mild to moderate number of TDP43-IR DN and NCI in the neocortical and predominantly subcortical regions, with occasionally neuronal intranuclear inclusions.[127, 132]

VCP

Valosin-containing protein (*VCP*), also known as p97, is a member of ATPase Associated with diverse cellular Activities protein family, and is involved in multiple cellular processes including protein degradation via ubiquitin proteasome system, cell division, DNA repair.[133-136] The first reported *VCP* mutations was identified in families with inclusion body myopathy with Paget's disease of the bone and frontotemporal dementia (IBMPFD).[137] IBMPFD is characterized by proximal and distal muscle

weakness resembling a limb-girdle dystrophy syndrome, Paget disease of bone and frontotemporal dementia.[138] To date, more than 15 mutations have been identified in *VCP*, mainly in CDC48 and D1 domains.[17] The frequency of *VCP* mutations is around 1-3% in FTD and FTD-ALS cohorts.[139, 140]

Large variation in phenotype has been observed among mutation carriers, even for patients within the same family.[138, 141, 142] About 90% of the patients presented with muscle weakness, 42% with Paget disease of the bone, and 30% with FTD.[143] ALS occurred in up to 10% of the mutation carriers. Due to the large variation in phenotype, the term multisystem proteinopathy (MSP) has been introduced to describe a combination of two or more phenotypes including IBM, Paget disease of the bone or ALS/FTD.[144] In the affected tissue, RNA binding protein (e.g. TDP-43, hnRNPA1, and hnRNPA2B1) or protein involved in ubiquitin-dependent autophagy proteins (e.g. p62/SQSTM1, VCP, optineurin, and ubiquilin-2) could be found.

The neuropathology is consistent with FTLD-TDP type D characterized by abundant TDP-IR NII and DN.[80] NCI are sporadically found in the neocortex.

SQSTM1

Sequestome 1 (*SQSTM1*), encoding for p62, is a multifunctional protein with multiple domains involved in cell survival and cell death.[145] It has also been reported that *SQSTM1* has an important role in targeting ubiquitinated proteins for degradation by autophagy or by proteasome pathways. Protein aggregates containing p62 is a hallmark of various neurodegenerative diseases, and has been suggested to be caused by an impaired autophagy through lysosomal dysfunction. Mutation in *SQSTM1* had been initially reported as genetic cause of Paget disease of bone, but several studies have reported *SQSTM1* mutations in patients with FTD and/or ALS considering *SQSTM1* as a genetic cause for MSP.[146-150] It explains 2 to 3% of FTD cases, and 3.8% of the familial FTD cases.[148, 149] BvFTD is the main presenting phenotype,[148, 149] although atypical initial presentation such as apraxia of speech and memory impairment has also been reported.[151, 152] Neuropathological findings consistent with FTLD-TDP type A or type B has been reported in a *SQSTM1* mutation carriers.[153]

New FTLD genes in next-generation sequencing era

CHCHD10

Coiled-coil-helix-coiled-coil-helix domain-containing protein 10 (*CHCHD10*) is involved in mitochondrial cristae morphology and mitochondrial DNA stability.[154] Mutation in *CHCHD10* (p.Ser59Leu) has initially been identified in a family with various clinical phenotypes including FTD-like syndrome, MND, cerebellar ataxia and mitochondrial myopathy. The frequency of *CHCHD10* mutation in FTD and ALS among European

cohorts varies from 0.7 to 3.5%.[155-158] A higher frequency up to 7.7% is observed in Chinese patients with FTD.[159] Although distinct rare variants in *CHCHD10* have been reported in patients with FTD and/or ALS, some variants have also been found in non-demented controls raising the question whether these variants were pathogenic or not fully penetrant.[155, 158, 160]

hnRNPA1 and hnRNPA2B1

A third genetic cause of MSP are mutations in heterogeneous nuclear ribonucleoproteins A1 (*hnRNPA1*) and heterogeneous nuclear ribonucleoproteins A2B1 (*hnRNPA2B1*), which segregated in two families with MSP.[161] These two genes encode for RNA binding proteins, and are involved in nucleic acid processing such as splicing regulation.[162] The identified mutations were mainly clustered in prion like domain which is involved in the biogenesis of various membraneless organelles. Mutations in *hnRNPA1* and *hnRNPA2B1* are assumed to be a rare genetic cause for FTD and/or ALS as various studies had failed to identify any mutations in these genes.[161, 163-166]

TBK1 and OPTN

The association of loss of function mutations in TANK-binding kinase 1 (*TBK1*) with sporadic ALS cases was discovered in a large case-control exome sequencing study.[167] An independent study confirmed this association with familial ALS cases and FTLD-ALS cases, and has evidenced by co-segregation of loss of function (LoF) *TBK1* mutations in a large ALS-FTD family.[168] Several other studies have replicated the association of LoF *TBK1* mutations with FTD and/or ALS,[169-172] but it remained unclear for missense variants due to absence of co-segregation with disease and borderline genetic association.

TBK1 LoF mutations result in 50% loss of TBK1 levels suggesting haploinsufficiency as a possible disease mechanism.[168, 171] In addition to LoF variants, missense variants located close to CCD2 domain of *TBK1* has frequently been observed in ALS-FTD phenotype, and has been hypothesized to affect the binding with optineurin (*OPTN*), a gene that linked to ALS and FTD.[168, 170] The frequency of LoF mutation in *TBK1* in FTD and FTD-ALS is estimated to be 1.1-1.8%,[169, 170, 172] and a higher frequency of up to 10% among FTD-ALS cases.[170, 173] Clinical presentation includes behavioral changes, frequently co-occur with ALS symptoms, but early memory impairment has also been reported.[174] Neuropathological findings consistent with FTLD-TDP type A or type B have been reported in *TBK1* mutation carriers.[169, 171, 174, 175]

OPTN is involved autophagy, and is regulated by TBK1 through phosphorylation.[176] The contribution of *OPTN* mutations is small in FTD,[177, 178] and only a few studies reported compound heterozygous variants in *OPTN* as a cause of FTLD-TDP type A or FTD-ALS.[171, 177] Interestingly, one FTLD-TDP case carried a deletion in *OPTN* and nonsense mutation in *TBK1*, suggesting a possibly interaction of these genes in the cause of

FTD.[171] This report underlined the contribution of oligogenic genes involving in the disease phenotype.

Genes with unclear significance in FTLD

A few genes that are associated with ALS, have also been implicated to cause FTD. Two of these genes are ubiquilin-2 (*UBQLN2*) and *FUS*, which have been reported to be involved in the pathogenesis of ALS and FTD.[179, 180]

Mutations in *UBQLN2*, an ubiquitin-like protein, have been identified in X-linked dominant ALS and ALS-FTD cases,[179] but its contribution to FTD is unclear. Only a few studies have reported variants in *UBQLN2* in pure FTD phenotype, but all outside the frequently mutated PXX repeat domain without supporting co-segregation in families or functional experiments.[181-183]

Mutations in *FUS* have been identified as genetic cause of ALS,[184, 185] with a mutation frequency of 5% in familial ALS and 1% in sporadic ALS.[125] Most of the mutations are located in the C-terminal, predominantly in familial cases, although mutations in the N-terminal have also been reported. The contribution of *FUS* mutations FTLD is uncertain, as only a few studies have reported *FUS* mutations without neuropathological support.[180, 186, 187] FUS is localized in the nucleus and is involved in RNA binding, splicing and nucleo-cytosolica RNA transport, which is similar to TDP43.[125] It is important to note that FUS pathology could be found in both ALS and FTLD, but *FUS* mutations have only been observed in exclusively ALS-FUS but not in FTLD-FUS.[79, 184, 185, 188] On the contrary, TAF-15 and transportin 1 (TRN1) positivity are found in the neuronal inclusions of FTLD-FUS patients, but not in ALS-FUS patients.[189] Additionally, FTLD-FUS proteins has been shown to be hypomethylated compared to ALS-FUS proteins.[190] These distinct features underlined a distinct disease mechanism between FTLD-FUS and ALS-FUS.

Genetic Risk factor

In addition to monogenic cause of FTD, several studies have reported genetic risk factor for FTD including variants in transmembrane protein 106B (*TMEM106B*), Ras-related protein Rab-38/cathepsin C (*CTSC/RAB38*), *TREM2* and known FTD genes (*MAPT* and *GRN*).[191-194] Of those, *TMEM106B* is the most replicated risk factor in FTD, particularly among *GRN* carriers.[193-199] The modifying effect of one SNP (rs1990622) in *TMEM106B* has been consistently replicated in *GRN* mutations carriers,[195, 196, 198] in which minor C allele of rs1990622 conferred a lower risk, whereas the more common T allele is associated with an increased risk of FTD. Interestingly, a lower median age at onset of 13 years has been reported for carriers of homozygous T allele of rs1990622 compared to carriers of heterozygous and homozygous C allele.[195]

The function of *TMEM106B* and its relation with GRN is not fully known. Several studies indicated that *TMEM106B* is a lysosomal protein involved in lysosomal size, function and lysosomal stress response.[200-203] The functional impact of *TMEM106B* on GRN has been supported by the finding of lower plasma GRN levels in *GRN* carriers and non-demented controls carrying the risk allele compared to protective allele.[195, 196] Furthermore, more than 2.5 times higher expression of TMEM106B in the frontal cortex in FTLD-TDP cases compared to unaffected controls.[194] In addition to its modifying effect of *GRN* mutation carriers, a protective effect of *TMEMB106B* has also been found in FTD and FTD-ALS patients with *C9orf72* expansions carriers.[204, 205]

Scope of this thesis

Genetic factors play a key role in the etiology of AD and FTLD. Both risk modifying common variants and highly penetrant rare variants could contribute to the development of AD and FTD. Recent development in next generation sequencing enabled us to further investigate the contribution of rare variants in these diseases. The distinct genetic, clinical and pathological features underlined the presence of different pathomechanisms. The study of genetic factors gives us more insights in the disease process, which is essential for the development of disease modifying drugs.

The aim of this study is to describe the contribution of rare variants in AD and FTLD using whole exome sequencing, and expands the mutation spectrum of these diseases. Furthermore, we aimed to describe the clinical and pathological features of these mutations carriers. The thesis is divided in two major parts:

2. Genetics of Alzheimer's disease

Chapter 2.1 describes the clinical and pathological features of patients with *PSEN1* and *PSEN2* mutations including two novel mutations. In Chapter 2.2, we describe rare variations in *EIF2AK3* gene in Dutch patients with AD and their pathological features.

3. Genetics of frontotemporal lobar generation

In chapter 3.1, we report two novel and one known *VCP* mutations in three patients with pure FTD as phenotype. Chapter 3.2 describes the finding of a rare variant in *TUBA4A* segregating in a family with FTD. In chapter 3.3, we report clinical and pathological findings of a large family with FTD and parkinsonism caused by mutation in *PRKAR1B* gene. In chapter 3.4, we estimate the contribution of *PRKAR1B* gene in an early-onset dementia cohort.

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CHAPTER 2

2

Genetic heterogeneity in Alzheimer's disease

CHAPTER 2.1

2.1

Genetic screening in early-onset Alzheimer's disease identified three novel presenilin mutations

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Abstract

Mutations in presenilin 1 (*PSEN1*), presenilin 2 (*PSEN2*) and amyloid precursor protein (*APP*) are major genetic causes of early-onset Alzheimer's disease (EOAD). Clinical heterogeneity is frequently observed in patients with *PSEN1* and *PSEN2* mutations. Using whole exome sequencing, we screened a Dutch cohort of 68 patients with EOAD for rare variants in Mendelian Alzheimer's disease, frontotemporal dementia and prion disease genes. We identified three *PSEN1* and two *PSEN2*. Three variants, one in *PSEN1* (p.H21Profs*2) and both *PSEN2* (p.A415S and p.M174I) were novel, and absent in control exomes. These novel variants can be classified as probable pathogenic, except for *PSEN1* (p.H21Profs*2), in which the pathogenicity is uncertain. The initial clinical symptoms between mutation carriers varied from behavioral problems to memory impairment. Our findings extend the mutation spectrum of EOAD, and underline the clinical heterogeneity among *PSEN1* and *PSEN2* mutation carriers. Screening for AD-causing genes is indicated in presenile dementia with an overlapping clinical diagnosis.

Introduction

Early-onset Alzheimer's disease (EOAD) accounts for 1-2% of all Alzheimer's disease (AD) cases. It can be caused by mutations in presenilin 1 (*PSEN1*), presenilin 2 (*PSEN2*) and amyloid precursor protein (*APP*) in an autosomal dominant inheritance pattern.[1-3] To date, more than 280 mutations have been found in *PSEN1*, *PSEN2* and *APP* (www.molgen.ua.ac.be/ADMutations).[4]

Presenilin 1 and presenilin 2 proteins are both key components of gamma secretases, which process APP by cleaving into Amyloid beta (Aβ) fragments.[5] Mutation in these genes impairs the proteolytic activity of gamma secretases, resulting in a disbalance of Aβ40 and Aβ42.[6] Considerable heterogeneity is found in the clinical presentation of *PSEN1* and *PSEN2* mutation carriers, including initial behavioral, language and dysexecutive problems, myoclonus, seizures, spasticity and hallucinations.[7, 8] The age at onset among mutation carriers also varies greatly, ranging from 23 to 71 years, even in families with the same mutation.[9] Neuropathologically, *PSEN1* mutation carriers often have more neuronal loss in the frontotemporal cortex than sporadic AD cases.[10] Furthermore, more neocortical senile plaques and higher Aβ42/Aβ40 plaque ratios are observed in *PSEN1* and *PSEN2* mutation carriers compared to sporadic AD.

In this study, we assessed the contribution of rare variants in Mendelian AD (*PSEN1*, *PSEN2* and *APP*), frontotemporal dementia (FTD) (*MAPT*, *GRN*, *TARDBP*, *VCP*, *CHMP2B*, *FUS* and *TBK1*) and prion disease genes (*PRNP*) in a Dutch cohort of 68 patients with EOAD using whole exome sequencing (WES). We found three novel and two reported variants in *PSEN1* and *PSEN2*. We describe the clinical and available pathological features of the *PSEN1* and *PSEN2* variant carriers.

Methods

Subjects

Patients were included either by referral to the Erasmus Medical Center department of neurology (Rotterdam, the Netherlands) or by visits to (nursing) homes. Patients underwent a clinical examination, neuropsychological assessment, neuroimaging and, if indicated, a lumbar puncture. The diagnosis probable AD was established according to the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria for AD.[11] EOAD was defined as an age at onset of ≤ 65 years. Family history was defined as positive if the patient had at least one first degree relative with dementia. Cerebrospinal fluid profile (CSF) consistent with AD was defined as amyloid beta 42 <550 pg/mL, Tau >375 pg/mL, and pTau>53 pg/ml or a tau/Aβ42 >0.52.[12] We selected 68 patients with EOAD for WES based on their initial clinical diagnosis of probable AD and/or CSF

profile consistent with AD, as mentioned above. CSF was present in 41 patients, and pathological diagnosis of AD was found in four patients.

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

Genetic analysis

DNA from all samples was prepared with the Illumina TruSeq Paired-End Library Preparation Kit, and 100 base pair paired-end reads were acquired by sequencing the libraries on a HiSeq 2000. Exomes were captured using Nimblegen Seqcap EZ Exome Capture Kit v2. All data were generated at the Human Genomics Facility (HuGeF; www.glimdna.org) at Erasmus Medical Center Rotterdam. Sequencing reads were aligned to the hg19 human genome assembly using BWA-MEM (version 0.7.3a),[13] followed by duplicate marking and sorting alignments by Picard Tools (version 1.9).[13] Subsequently, Genome Analysis Tool Kit (GATK) (version 3.3) was used to perform indel realignment, base quality score recalibration, and variant calling.[14] Subsequently, we used Variant Quality Score Recalibration from GATK to filter out low-quality variants using thresholds of 90 for Single nucleotide variants (SNV) and 50 for indels. All individuals in the WES data were checked for sex concordance using Plink.[15] Variants were annotated using ANNOVAR.[16]

We focused on missense, nonsense, splicing and frameshift variants in Mendelian AD (*PSEN1*, *PSEN2* and *APP*), frontotemporal dementia (FTD) (*MAPT*, *GRN*, *TARDBP*, *VCP*, *CHMP2B*, *FUS* and *TBK1*) and prion disease genes (*PRNP*) as described in previous studies.[17, 18] Any identified variants with a minor allele frequency ≥0.1% in the Genome aggregation database (gnomAD), Healthy EXomes (HEX), Genome of the Netherlands, and in-house WES data from the Rotterdam Study, were filtered out.[19-22] Subsequently, we interpreted the identified variants using the Alzheimer Disease & Frontotemporal Dementia Mutation Database (www.molgen.ua.ac.be/admutations/)[4] and AlzForum (www.alzforum.org/mutations) databases, and a literature search. Combined Annotation Dependent Depletion score (CADD) was used to predict the pathogenicity of the variants.[23] Variants in *PSEN1* and *PSEN2* were further classified according to the algorithm described by Guerreiro et al.[24] All identified variants were confirmed by Sanger sequencing. Screening of chromosome 9 open reading frame (*C9orf72*) repeat expansions was performed on selected cases with upper and/or lower motor neuron signs or a family history positive for motor neuron sign.

Histology and immunohistochemistry

The Netherlands Brain Bank performed brain autopsy according to their Legal and Ethical Code of Conduct. Tissue blocks taken from all cortical areas, hippocampus, amygdala, basal ganglia, substantia nigra, pons, medulla oblongata, cerebellum, and

cervical spinal cord, were embedded in paraffin blocks and subjected to routine staining with hematoxylin and eosin, periodic acid-Schiff reaction, and silver staining. The slides were also immunochemically stained with Anti-β-Amyloid, 1-42 (Biolegend, 12F4, dilution 1:400), Anti-β-Amyloid, 1-40 (Biolegend, 11A50-B10, dilution 1:400), α-synuclein (Novocastra, NCL-ASYN; dilution 1:10000) and AT8 (Thermo Fisher Scientific, MN1020; dilution 1:200). Braak stage was ascertained according to the revised National Institute on Aging-Alzheimer's Association guidelines.[25]

Results

The mean age at onset in our cohort of 68 EOAD cases was 57.7 years (range 51-65). A positive family history was found in 39 cases (57%).

Mutation screening

After filtering, we found five rare variants, three in *PSEN1* and two in *PSEN2* (Table 1). Two variants in *PSEN1* were previously described in EOAD cases (p.A79V and p.P264L).[26-28] One single *PSEN1* (p.H21Profs*2) (Figure 1) and both *PSEN2* (p.A415S and p.M174I) were novel. No rare variants were found in *APP* gene. All variants, except one in *PSEN1* (p.A79V), were unknown in gnomAD, HEX, GoNL and exome data from the Rotterdam Study. No rare variants in *SORL1*, *TREM2* or *ABCA7* have been found in the five *PSEN* carriers. The main clinical features of the *PSEN1* and *PSEN2* mutations carriers are summarized in Table 2.

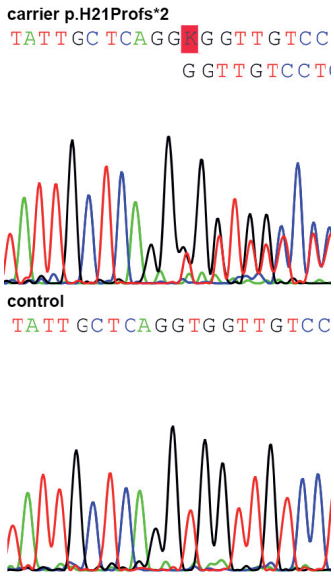

Figure 1. Electropherogram of *PSEN1* p.H21Profs*2 variant.

Table 1. Rare variants in *PSEN1* and *PSEN2* identified in the EOAD cohort

Variant interpretation	Gene	Exon	Nucleotide change	Protein change	Status	Protein domain	GnomAD	CADD Phred score
Known pathogenic								
	PSEN1	4	c.236C>T	p.A79V	rs63749824	N-terminal	$1.44x10^{-02}$	33
	PSEN1	8	c.791C>T	p.P264L	rs63750301	HL-VI a	$4.08x10^{-03}$	35
Probable pathogenic*								
	PSEN2	7	c.522G>A	p.M174I	Novel	HL-I	0	20.8
	PSEN2	13	c.1243G>T	p.A415S	Novel	TM-IX	0	33
Variant of unknown significance								
	PSEN1	3	c.62delA	p.H21Profs*2	Novel	N-terminal	0	NA

EOAD, early-onset Alzheimer's disease; PSEN1, presenilin 1 (NM_000021); PSEN2, presenilin 2 (NM_000447); HL, hydrophilic loop; TM, transmembrane; GnomAD, genome aggregation database; CADD, Combined Annotation Dependent Depletion score; NA, not available. * Pathogenicity classification based on the algorithm proposed by Guerreiro et al. (2010).

Table 2. Clinical characteristics of the mutation carriers in *PSEN1* and *PSEN2*

Gene	Protein change	Diagnosis	AAO	Family history	Initial presentation	Behavioral symptoms*	Myoclonus	Seizure	delusions/ hallucinations	Spasticity	Extrapyramidal sign
PSEN1	p.H21Profs*2	AD	60	positive	memory impairment	no	no	no	no**	no	no
PSEN1	p.A79V	AD/DLB	64	positive	behavioral changes	suspicious	no	no	yes	no	yes
PSEN1	p.P264L	AD/ FTD+PLS	56	positive	gait disturbance	labile affect	no	no	no	yes	no
PSEN2	p.M174I	AD/FTD	51	positive	memory impairment	depressed	yes	yes	yes	yes	yes
PSEN2	p.A415S	AD	59	negative	gait disturbance	no	yes	no	yes	yes	yes

AAO, age at onset; AD, Alzheimer's disease; DLB, Dementia with Lewy bodies; FTD, frontotemporal dementia; PLS, Primary lateral sclerosis; *PSEN1*, presenilin 1; *PSEN2*, presenilin 2. Symptom presentation referred to the clinical presentation at disease onset, * Behavioral symptoms at first visit ** Nightmares treated with Risperidone.

Novel *PSEN1* mutation p.H21Profs*2

PSEN1 p.H21Profs*2 is located in exon 3 which codes for the N-terminal domain. The female carrier, aged 60, presented with progressive memory impairment, followed by problems in orientation, housekeeping, verbal expression, and loss of initiative. Neurological examination one year after onset showed a Mini Mental State examination (MMSE) of 23/30, mild bradyphrenia, and left-sided cogwheel sign during risperidone medication prescribed for nightmares. Neuropsychological assessment showed deficits in multiple domains including memory, executive functions, and visual perception, however several tests were prematurely ended due to poor performance. MRI showed global brain atrophy and extensive white matter lesions in the parieto-occipital region. During follow up, the patient was treated with acetylcholinesterase inhibitors until death, aged 65. Family history revealed a mother with dementia with an age at onset of 60.

Novel *PSEN2* mutation p.M174I

The p.M174I variant in the *PSEN2* gene was identified in a female patient who developed memory problems at the age of 53. This variant is located in the hydrophilic loop domain of the presenilin 2 protein. The patient developed progressive anomia and memory impairment within the first three years. She had a depressed mood and was apathetic. Neurological examination (two years after onset) showed an MMSE score of 18/30, short-term memory impairment, dyscalculia, bradyphrenia, and apraxia. Over time, the patient developed behavioral changes, including aggression, restlessness, obsessive thinking, blunted emotions, and binge eating. MRI showed generalized cerebral atrophy most prominent of the frontal lobes; CSF was not available. The clinical diagnosis was either presenile AD with frontal presentation or a behavioral variant of frontotemporal dementia (bvFTD). After admission to a nursing home, she developed hallucinations, seizures, spasticity and mutism. She died from pneumonia aged 64. The patient's mother had been diagnosed with AD at the age of 75.

Neuropathological examination of p.M174I *PSEN2* carrier revealed severe neuronal loss and gliosis of the frontal, temporal and parietal cortices, cornu ammonis (CA) region 1 of the hippocampus and to a lesser extent in the other CA regions, subiculum and occipital cortex. Abundant AT8-positive threads and tangles accompanied by many Aβ-positive senile plaques of variable size and morphology were found in the neocortex, predominantly in the frontal cortex, and to a lesser extent in parietal and temporal cortices and CA1 of the hippocampus (Figure 2A-E). Classical, predominantly Aβ42-positive plaques with a dense core were seen in 5-10% of the plaques. Only a small number of plaques were stained Aβ40-positive (Figure 2B and C). The severe involvement of the frontal, temporal and parietal cortices is consistent with advanced stage AD, Braak stage 6, A3B3C3.[25] Furthermore, many α-synuclein positive Lewy bodies (LB) were seen (Figure 2F), especially in the amygdala and parahippocampal cortex, and a few in the substantia nigra and brainstem.

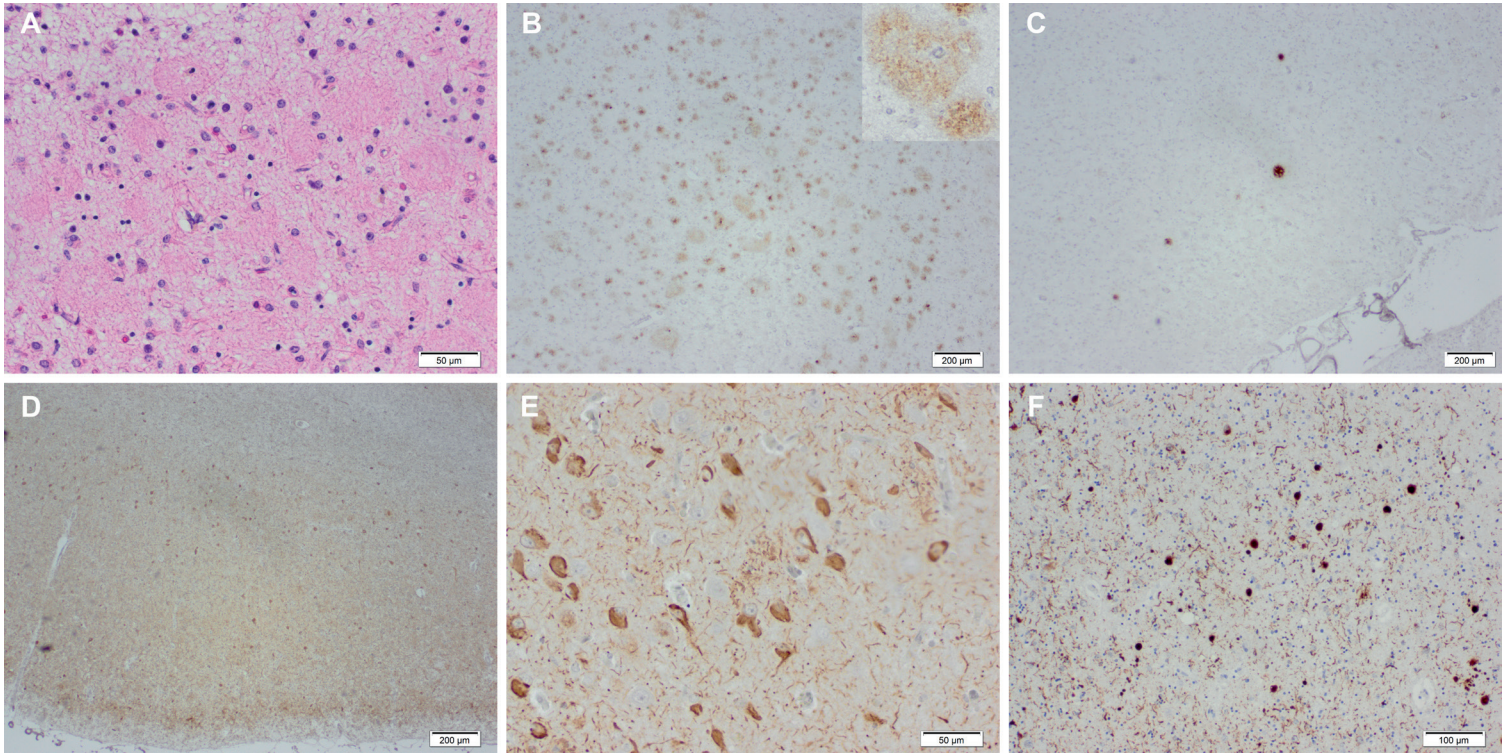

Figure 2. Pathological features of *PSEN2* p.M174I mutation carrier. Histological section and immunostaining in the frontal cortex and hippocampus of *PSEN2* p.M174I carrier. Hematoxylin and eosin staining of the frontal cortex showed many plaques and eosinophilic bodies **(A)**. Extensive numbers of Aβ42 immunoreactive (IR) plaques were observed in all layers of the frontal cortex **(B)**, while only small numbers of Aβ40-IR plaques were found **(C)**. Immunostaining with tau protein showed many AT8-IR tangles and small threads in the frontal **(D)** and hippocampus **(E)**. Numerous Lewy bodies positive for α-synuclein were seen in the amygdala **(F)**.

Novel *PSEN2* mutation p.A415S

The *PSEN2* (p.A415S) variant, located in the transmembrane IX domain, was identified in a female patient aged 59 who presented with memory impairment and gait disturbance. She developed difficulties in verbal expression with problems in word finding, hallucinations and myoclonus. Her grandmother, aunt and uncle also had dementia, all with onset age > 65. Neurological examination revealed an MMSE of 17/30, hyperreflexia in the arms and legs, and a spastic gait. Neuropsychological assessment showed impairment in memory, language, perceptuospatial skills, and praxis. MRI showed global cerebral and cerebellar atrophy. Her CSF was compatible with an AD profile with decreased amyloid beta (203 pg/ml), increased phospho-tau (95 pg/ml) and total tau (551 pg/ml). Cognitive functions including verbal expression and motor functioning deteriorated over the following three years, and she was admitted to a nursing home. At a later stage, aged 65, she became mutistic and wheelchair bound.

Previous reported *PSEN1* mutations p.A79V and p.P264L

The patient with *PSEN1* p.A79V mutation presented with cognitive impairment, delusions, hallucinations and parkinsonism suggestive for probable dementia with Lewy bodies (DLB) when aged 64.[29] Family history revealed five siblings and a father who had AD before the age of 65. Three of the affected siblings also suffered from hallucinations. DNA was only available in one sister with AD and onset-age 74 who also carried the *PSEN1* p.A79V mutation.

The second mutation, *PSEN1* p.P264L, was found in a patient who presented with gait disturbance aged 56. Memory impairment, behavioral changes and impairment in word comprehension and word finding were also observed early in the disease process. The clinical diagnosis was suspected bvFTD or a frontal variant of AD with primary lateral sclerosis. The patient's grandmother, mother and one sister also had dementia. Screening on *C9orf72* was negative.

Discussion

We identified one novel frameshift deletion in *PSEN1* (p.H21Profs*2) and two novel missense in *PSEN2* (p.A415S and p.M174I) using WES in a Dutch cohort with EOAD. These novel variants were not present in the public sequencing database and population-match exomes. Additionally, we found two known *PSEN1* variants (p.A79V and p.P264L), reported as pathogenic in previous studies.[31] The *PSEN1* and *PSEN2* variant carriers had variable clinical presentation including memory impairment, behavioral changes, pyramidal and extrapyramidal symptoms.

The first novel variant, *PSEN1* p.H21Profs*2, is a frameshift mutation resulting in a premature stop codon in the sequences of exon 3, and as a consequence, a truncated protein. Frameshift deletions in *PSEN1* and *PSEN2* were reported as a possible genetic cause of EOAD, however, the pathogenicity is debatable.[7, 18, 30] Variable phenotypes of the frameshift deletion mutation carriers including AD, mild cognitive impairment, frontotemporal dementia and amyotrophic lateral sclerosis have been reported. Segregation or functional studies supporting the pathogenicity of these mutations are scarce. One study reported a reduced presenilin 2 protein expression in the lymphoblast cells of a *PSEN2* (p.G359Lfs*74) mutation carrier compared to control, but a reduced presenilin 2 protein expression was also found in a mutation carrier with autopsy confirmed frontotemporal lobar degeneration.[18] We classified the pathogenicity of our novel variant as a variant of uncertain significance, as we were unable to investigate the functional effect due to the lack of additional blood or brain tissue from the patient.

The second novel variant, *PSEN2* p.M174I, was classified as probable pathogenic according to the algorithm proposed by Guerreiro et al.[24] based on reported mutation in the same codon (p.M174V) and the altered Aβ level at neuropathology. However, conflicting results about the pathogenicity of p.M174V have been reported.[24, 31-33] Although the pathogenicity of *PSEN2* p.M174V is unclear, our case carried a different *PSEN2* variant with a high CADD score of 20.8, which is unknown in gnomAD. Furthermore, Our case's high number of neocortical senile plaques with a higher Aβ42/Aβ40 ratio are consistent with the reported pathological features of *PSEN1* and *PSEN2* mutations carriers;[10] therefore, this variant may be causative for AD.

The third novel variant, *PSEN2* p.A415S, was classified as probable pathogenic based on: 1) conserved residues in *PSEN1* A431; 2) previously reported mutations in the same codon (A431E and A431V);[34, 35] 3) CSF profile with low amyloid beta and high phospho- and total tau indicative for AD. Furthermore, the damaging effect of this variant was also supported by a high CADD score of 33.

The atypical AD symptoms of the mutation carriers in our study have been frequently reported in *PSEN1* carriers.[8] Up to 16% of *PSEN1* carriers had an atypical presentation, and about 8% of *PSEN1* mutations carriers presented with behavioral changes at onset. Similar to previous reports, the *PSEN1* p.P264L carrier in our study also had symptoms consistent with spastic paraparesis.[36] Spastic paraparesis is present in 25% of *PSEN1* cases, and is frequently associated with mutations beyond codon 200.[8, 37]

Additionally, three of the five carriers in our study had delusions or hallucinations during the disease course. In one (*PSEN1* p.A79V) carrier, the clinical diagnosis was suspect for DLB[29] or AD due to the presence of hallucinations and parkinsonism together with memory impairments. Interestingly, three siblings of this carrier also had hallucinations, but prominent cognitive impairment made the clinical diagnosis of AD

more likely. The association of *PSEN1* p.A79V with DLB as phenotype has been reported previously.[38] Notably, DLB phenotype has also been reported in a patient with *PSEN2* p.A85V mutation, which is homolog to *PSEN1* p.A79V.[39] Neuropathological examination of the *PSEN2* p.A85V carrier showed abundant neocortical LB as well as AD pathology, suggesting a link between this mutation and LB pathology. Up to 96% of LB pathology has been reported in familial AD with *PSEN1* mutations, while lower percentages have been reported in AD with *PSEN2* mutation (64%) and sporadic AD (60.7%).[40, 41] However, this difference in LB pathology between these groups has not been confirmed in other studies.[42, 43] Unfortunately, no brain tissue of the *PSEN1* p.A79V carrier was present to examine the presence of LB pathology. Another possibility for the DLB phenotype in our case may be the presence of another gene mutation for DLB. Recent studies identified the genetic association of variants in the glucocerebrosidase (*GBA*) gene with DLB.[44-46] In our cases, screening of *GBA* did not identify any rare variants in this gene (data not shown). Nevertheless, the presence of unidentified genetic factor(s) contributing to the DLB phenotype cannot be ruled out.

We used exome sequencing to screen for mutations in known dementia genes. Although we were able to identify small deletion and missense mutations using this method, large deletion such as DeltaE9 in *PSEN1*,[47] *C9orf72* repeat expansions, and copy number variations variants can be missed, and thus we may underestimate the frequency of the mutations. However, these mutations in *PSEN1* and *PSEN2* are rare, and the observed frequency of *PSEN* variants in 7% of our cohort of 68 EOAD is in line with those reported earlier by Brouwers et al.[48] Another limitation is that the follow-up time in most patients was limited to a few years, and we cannot rule out that additional symptoms may have developed later during their disease course. Lastly, DNA was only available from a sister with AD of the p.A79V mutation carrier; we were unable to include other family members of the other mutation carriers. Therefore, segregation analysis could not be performed for these mutation carriers.

Our study provides further insights into the genetics of AD by identifying three novel mutations in *PSEN1* and *PSEN2*, and highlights the clinical heterogeneity of the presenilin mutation carriers. Although overlapping clinical diagnosis with FTD or DLB was found in some patients, we were unable to identify other probable pathogenic dementia-causing genes besides *PSEN1* and *PSEN2*. Genetic screening of AD causative genes is valuable in patients with clinically suspected EOAD with atypical clinical features, particularly in familial cases.

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CHAPTER 2.2

2.2

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 towards association with AD (*p*-value 0.1). Immunohistochemical staining with pPERK and peIF2α of three *EIF2AK3* AD carriers showed an increase in hippocampal neuronal cells expressing these proteins compared to non-demented controls, but no difference was observed compared to AD non-carriers. This study suggests that rare variants in *EIF2AK3* may be associated with disease risk in AD.

Introduction

Alzheimer's disease (AD) is the most common cause of dementia, characterized by progressive decline in memory and other cognitive functions.[1] Genetic factors are strongly linked to AD, and in about 5% of cases an autosomal dominant mode of inheritance has been reported.[2] 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;[3-7] this accounts for approximately 13% of early-onset AD.[8] In late-onset AD, the ε4 allele of apolipoprotein E gene have been found to be the most common risk factor.[9]

Neuropathologically, the aggregation of misfolded proteins is a major hallmark of many neurodegenerative disorders.[10] The accumulation of extracellular amyloid plaques and intracellular neurofibrillary tangles are the hallmarks of AD.[11] 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.[10, 12] The UPR is induced by three transmembrane proteins in the ER: protein kinase RNA-like endoplasmic reticulum kinase (PERK), Inositol Regulating Enzyme 1 (IRE1) and Activating Transcription Factor 6 (ATF6). Activation of UPR lead to transient suppression of protein synthesis and increased expression of genes aimed to restore the homeostasis of the ER.[10] Pharmacological and genetic manipulation of the UPR pathways in animal studies, in particularly the PERK pathway, has been reported to inhibit neurodegeneration.[13]

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.[14-18] Furthermore, a large exome micro-array study identified rare coding variants in *PLCG2*, *ABI3* and *TREM2*, explaining a small part of missing heritability in AD.[19] These studies indicate the existence of other rare variants related to the heritability of AD.

In this paper, we performed WES in eight 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 two of these families. Together with previous reports on an increased activation of PERK in AD brain and the involvement of PERK in memory and learning,[20] these findings suggest the possible role of *EIF2AK3* in the pathogenesis of AD.

Materials and Methods

Subjects

Our discovery dataset included 19 AD patients from eight Dutch families with a high AD burden. Each family had at least two patients with AD suggestive of an autosomal dominant inheritance pattern, except one 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). Non-demented 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 two patients with AD from each family. Non-demented family members with a minimum age of 65 were used to test for segregation in their respective family.

Table 1. Baseline characteristics of the families

Family	Cases	Controls	WES cases	Mean age at onset (range)	Mean age at last visits controls (range)	% female	APOE fraction ε2/ ε3/ ε4
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

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 contains 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. AD, Alzheimer's disease; WES, individuals selected for whole exome sequencing; NA, not available.

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.[21]

To replicate the association of our candidate gene with AD, we used exome data available from 547 AD cases and 1070 controls from three different sites (the Rotterdam

Study, Amsterdam Dementia Cohort (ADC-VUmc) and Alzheimer Centrum Erasmus MC (AC-EMC)) included from a Dutch AD exome dataset, previously described by Holstege et al.[18] We then genotyped our candidate variant in 1055 AD cases and 6162 controls from the Rotterdam study;[22] 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.

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 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 with the Illumina TruSeq Paired-End Library Preparation Kit, and 100 base-pair paired end reads were acquired by sequencing the libraries on a HiSeq 2000. For the Dutch exome dataset, 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)[23], and Picard Tools (version 1.9)[24] 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.[25] Haplotype Caller from GATK was used to create gVCF files, and to call variants from these gVCF files. For the exome data from the eight families (discovery set), we used hard filters according to GATK best practices to filter out low quality variants. For the exome data from the three Dutch cohorts, we used Variant Quality Score Recalibration (VQSR) with >99% sensitivity to filter out low-quality variants. Subsequently, Plink was used to calculate Principle component (PC), and outliers on the first two PCs were removed.[26] Related individuals with identity by decent value > 0.1 were also removed from the analysis set. All individuals in the WES data were checked for sex concordance using Plink.[26] Variants from all datasets were annotated using ANNOVAR.[27]

In our discovery set, we used a family based analysis to identify candidate genes from the eight 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).[28-32] If

the same variant or different variants in the same gene were identified in at least two families, these variants were selected as candidates for follow up and tested with Sanger sequencing for segregation in their respective families.

Sanger sequencing

We used Primer 3 [33] 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, CA, USA).

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 covariate in the secondary analysis.

Statistical analysis of the candidate genes in the Dutch exome dataset

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) ≤ 1% in ExAC was 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.

Histology and immunohistochemistry

The Netherlands Brain Bank performed brain autopsy according to their Legal and Ethical Code of Conduct. Tissue blocks of three *EIF2AK3* carriers (two from family NLAD 1 and one 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 haematoxylin 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, 1:12800) and phosphorylated eukaryotic initiation factor-2α (peIF2α) (SAB4504388, Sigma-Aldrich, St. Louis, MO, 1:100). We performed staining of pPERK and peIF2α on the frontal, temporal and hippocampal regions of our three pathological-confirmed AD *EIF2AK3* carriers, three AD non-carriers, and three non-demented controls. Immunohistochemical staining of the neurons with pPERK and peIF2α were scored with a semi-quantitative 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, ++++: high density of stained cells (> 30 percent) in almost every field of the section.[34,35] In the frontal and temporal regions, the average number of positive stained cells per field were counted in nine 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 peIF2α staining to calculate the percentage of stained neurons. We focused on 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 three 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 non-carriers. All tests are two-sided significant, and a *p*-value below 0.05 was assumed as being statistically significant.

Immunoblot analysis

Post-mortem fresh-frozen brain tissue of frontal cortex from three carriers of *EIF2AK3* mutations (III:15 and III:18 from family NLAD 1 and III:7 from family NLAD 4, Supplementary Figure 1) and three AD cases were extracted from the frontal cortex with buffers of increasing strength.[36] Briefly, grey matter was extracted at 5 ml/g (volume/weight) with low salt buffer (10mM Tris, pH 7.5, 5mM EDTA, 1mM DTT, 10% sucrose, and a cocktail of protease inhibitors), high salt-Triton buffer (low salt + 1% TritonTM X-100 + 0.5M NaCl), myelin floatation buffer (30% sucrose in low salt + 0.5M NaCl), and sarkosyl (SARK) buffer (1% N-lauroylsarcosine in low salt + 0.5M NaCl). The SARK insoluble material was extracted in 0.25 ml/g urea buffer (7M urea, 2M thiourea, 4% 3-[(3- cholamidopropyl) dimethylammonio]-1-propanesulphonate (CHAPS), 30mM Tris, pH 8.5). Proteins were resolved by 7.5% SDS-PAGE and transferred to PVDF 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).

Results

Family based exome analysis of the discovery set

In our discovery analysis of 19 AD patients from eight 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 eight 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 candidate as these are reported as frequent hitters in many WES datasets.[37] We selected the gene *EIF2AK3*, encoding for pancreatic endoplasmic reticulum kinase (PERK) as top candidate gene,[7] based on its involvement in memory and learning, and on its neurodegenerative role in AD and other neurodegenerative diseases,[12, 38]

The first *EIF2AK3* variant, p.R240H (rs147458427), was heterozygous in four affected individuals (including one with mild cognitive impairment) of family NLAD 1, and in one non-demented, 72-year old cousin of the proband (Supplementary Figure 1). This variant had a CADD score of 31 and a frequency of $8.00x10^{-04}$ in ExAC. The second *EIF2AK3* variant, p.N286S (rs150474217), had a low CADD score of 0.002 and a frequency of $3.00x10^{-05}$ in ExAC, and was confirmed in four patients with AD from family NLAD 4 and in one non-demented, 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 ε4*; the third patient was heterozygous for *APOE ε4*. All patients were diagnosed with early onset AD.

Sanger sequencing on the remaining variants in the 32 candidate genes shared among the eight 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.

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 dataset. We detected 23 *EIF2AK3* variants in this dataset (Figure 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 did not sustain the Bonferroni correction (Supplementary Table 3). We then performed a second analysis with *APOE* as additional covariate showing the frequency of *EIF2AK3*-carriers with at least one copy *APOE ε4* 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 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 to controls (OR=3.03; 95%CI 0.78-11.48, *p*=0.10), and an association with AD after adjusting for APOE as additional covariate (OR=2.57; 95%CI 0.69-9.51, *p*=0.16), however, in both cases the results were not statistically significant.

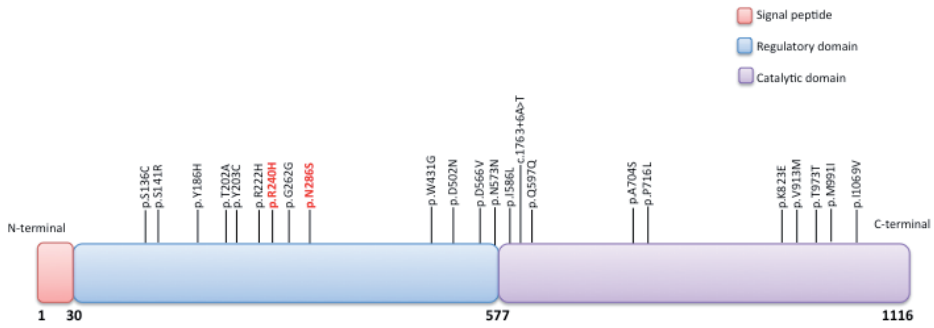

Figure 1. Schematic representation of *EIF2AK3* gene and relative position of the *EIF2AK3* variants found in the present study. The gene *EIF2AK3* contains 1116 amino acids and is composed of a signal peptide, a regulatory domain and a catalytic domain. Variants highlighted in red are found in the family based analysis.

Immunohistochemistry and immunoblot analysis

In our *EIF2AK3* carriers, many neurons with positive staining for pPERK and peIF2α 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 peIF2α staining in neurons were punctate shaped and were located in the cytoplasm, as reported in previous studies (Figure 2A-F).[34, 35] One carrier (III:18) from family 1 had severe neuronal loss in the CA regions and subiculum. Overall, the staining of peIF2α was more prominent than pPERK (Figure 2A,2D). All elderly non-demented controls showed a low to moderate degree of pPERK staining in the hippocampus. *EIF2AK3* carriers had significantly more positive staining than non-demented controls in the hippocampus (*p*=0.04) and temporal region (*p*=0.03). For peIF2α, a trend for more positive staining was only observed in the hippocampus of *EIF2AK3* carriers compared

Table 2. Scoring of inclusions for peIF2α and pPERK antibodies

				peIF2α			pPERK		
ID	Braak stage	Age at death	PMD	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 peIF2α and pPERK for carriers with *EIF2AK3* variants, Alzheimer's disease non-*EIF2AK3* controls and non-demented controls. -, negative; +, rare; ++, low density (up to 10%); +++, moderate density (11-30%); ++++, high density, >30%). AD, Alzheimer's disease; ND, Non-demented; PMD, Post mortem delay.

to non-demented 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 stages 6 with extensive tau pathology in the hippocampus, frontal, temporal and parietal cortices.

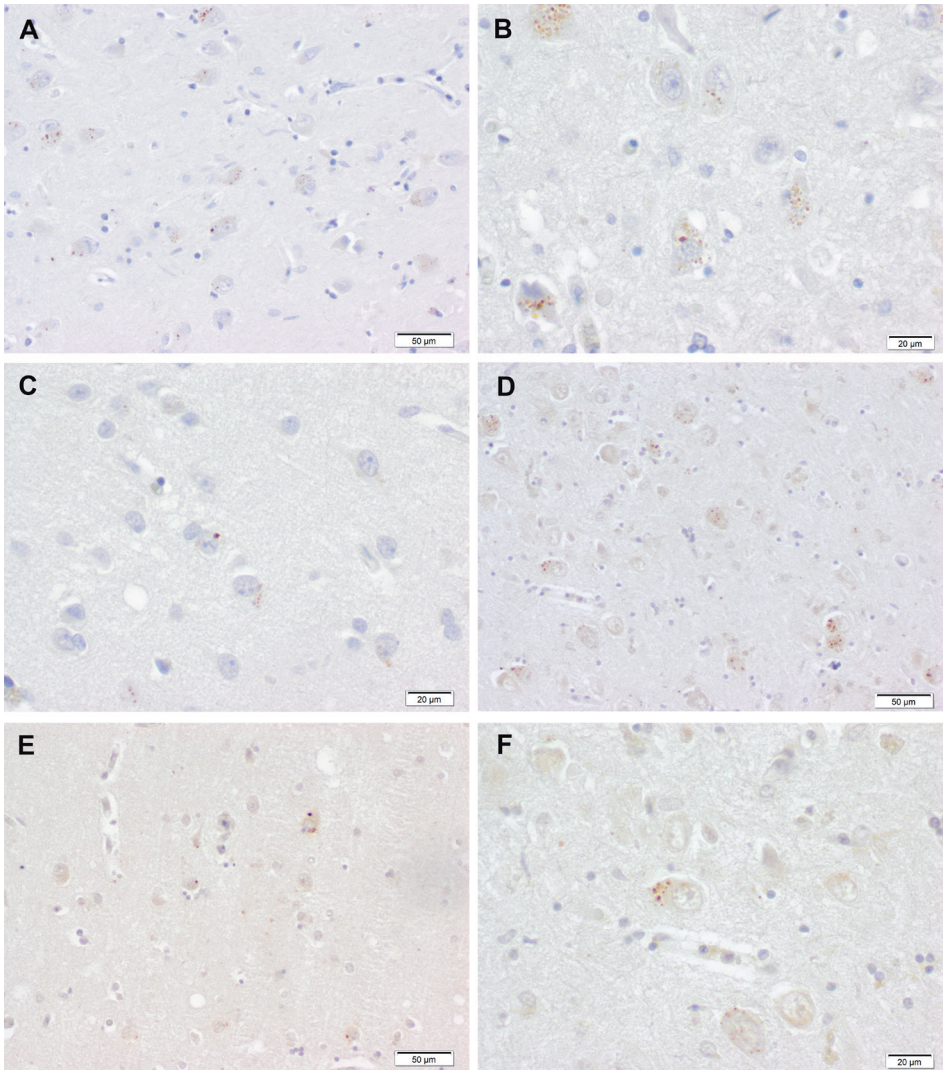

Figure 2. Immunohistochemical staining of pPERK and peIF2α in the AD cases with *EIF2AK3* mutations. Activated pPERK and peIF2α was found in the hippocampus and temporal regions **(A-F)**. High numbers of pPERK stained cells were observed in the cornu ammonis **(A)** and subiculum **(B)** of the hippocampus, and lesser numbers were found in the temporal cortex **(C)**. Abundant neurons with peIF2α staining were also found in the hippocampus **(D)**, and a moderate number of stained cells were found in the frontal cortex **(E)**. Cytoplasmic peIF2α staining is punctate shaped **(F)**, and is similar to the pPERK staining **(B)**. Scale bars have been added to the figures.

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 Figure 2).

Discussion

This is the first study to investigate the role of rare variants in *EIF2AK3* in patients with AD. We performed whole exome sequencing in eight Dutch families with a high burden of AD, and identified *EIF2AK3* as a candidate gene in two 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 peIF2α staining was more prominent in *EIF2AK3* carriers than in controls, it was similar to AD non-*EIF2AK3* carriers.

We identified two distinct variants in *EIF2AK3* segregating with AD in two 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 one SNP (rs7571971) in *EIF2AK3* was associated with AD in *APOE* ε4 carriers, but not independent of *APOE*,[39] 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 dataset 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 towards 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* ε4 carriers among the *EIF2AK3* carriers in the two families and in the Dutch AD exome dataset further support an association of *EIF2AK3* variant with AD in *APOE* ε4 carriers as indicated by Liu et al,[39] 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 unfolded protein response (UPR).[20, 40] Our hypothesis was that variants in *EIF2AK3* may enhance PERK signaling, resulting in increased phosphorylation of tau by glycogen synthase kinase 3β (GSK3β) and amyloidogenesis (by BACE1). Previous studies have indicated that PERK-eIF2α signaling is involved in the modulating of tau phosphorylation and APP

processing in AD,[35, 40, 41] but that it is also correlated with the level of tau pathology in Progressive Supranuclear Palsy and AD.[34, 35] pPERK immunoreactivity also colocalized with GSK3β in neuronal cells, which is involved in tau phosphorylation.[35, 41] Treatment with a PERK-inhibitor (GSK2606414) in transgenic mice with frontotemporal lobar degeneration and overexpression of p.P301L mutation resulted in reduced GSK3β-levels and tau phosphorylation compared to transgenic mice without PERK inhibitor treatment.[42] Moreover, *PSEN1* (5XFAD) mutated mice with PERK haploinsufficiency had lower levels of Beta-secretase 1 (BACE1) than those with normal PERK levels, resulting in lower amyloid-beta peptides levels and plaque burden, as well as fewer memory deficits and cholinergic neurodegeneration.[40] Reduced synaptic plasticity and spatial memory deficits were found in APP/PS1 AD model mice with PERK happloinsufficiency.[43] 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-eIF2α signaling in the *EIF2AK3* carriers is supported by the more positive staining of pPERK and peIF2α compared to non-demented controls, indicating an increased activation of UPR. This increased UPR has also been observed in AD and PSP patients in previous studies.[34, 44] However, we did not find any differences in pPERK and peIF2α 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 two 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.[14] 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) non-demented controls in some families to analyze segregation; some non-demented controls may still develop dementia at older age. Finally, the frequency of *APOE* ε4 is high in some families, and *APOE* ε4 segregates with the disease in some of them. This is also true for family 4, in which variant p.N285S was found; four patients and one individual with memory complaints carried at least one copy of *APOE* ε4. However, all four patients carrying p.N285S and *APOE* ε4 had early onset AD, indicating a possible additional effect of genetic variation in *EIF2AK3* on the risk of AD among *APOE* ε4 carriers, as indicated in a previous study.[39] 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 two families

and a gene-based analysis in the Dutch WES cohort. Immunohistochemistry confirmed more activation of UPR, characterized by increased pPERK and peIF2α in AD patients compared to non-demented controls, but not between *EIF2AK3* carriers and AD non-carriers. Further studies are needed to investigate the full contribution of rare variants in *EIF2AK3* in the development of AD.

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Supplementary Tables and Figures

Supplementary Table 1. Filtering step in each family with Alzheimer's disease

	Family 1 (*n*=3)	Family 2 (*n*=2)	Family 3 (*n*=3)	Family 4 (*n*=2)	Family 5 (*n*=2)	Family 6 (*n*=3)	Family 7 (*n*=2)	Family 8 (*n*=2)
Number of variants found	46692	48091	43748	44497	41752	43548	41575	40792
Missense/splicing/stop/frameshift indel	13289	13663	12196	12323	11742	12179	11580	11348
1000G EUR ≤0.5% and ESP EA op ≤0.5%	1724	1483	1133	1072	1065	1321	1088	948
ExAc NFE ≤0.5%	1110	884	517	513	509	729	558	425
GONL ≤0.5%	1085	858	500	479	480	701	539	405
RS cohort non-demented ≤0.5%	1048	829	479	439	441	657	512	375
Shared by cases	74	27	60	108	116	81	132	136

1000G EUR = 1000 genomes European; ESP EA = Exome sequencing projects European; ExAC NFE = Exome Aggregation Consortium Non-Finnish European; GoNL = Genome of the Netherlands; RS cohort = Rotterdam Study cohort; RS = Rotterdam Study.

Supplementary Table 2. Candidate variants from the family based analysis

Location	Gene	Variant information	ExAC NFE	Number of families	Sanger sequencing WES cases	Sanger sequencing in related cases and controls
chr9_100777705	ANP32B	ANP32B:NM_006401:exon7:c.G748A:p.D250N	0	5	not confirmed	NA
chr9_100777710	ANP32B	ANP32B:NM_006401:exon7:c.T753A:p.D251E	0	6	not confirmed	NA
chr15_51240316	AP4E1	AP4E1:NM_001252127:exon11:c.A1051C:p.I351L	0.0017	1	confirmed	Heterozygous in 1 available control
chr15_51285811	AP4E1	AP4E1:NM_001252127:exon17:c.A2110G:p.T704A	$3.01x10^{-05}$	1	confirmed	Heterozygous in 3 controls, but absent in 2 other cases
chr15_40627436	C15orf52	C15orf52:NM_207380:exon11:c.G1528T:p.G510X	0	2	not confirmed	NA
chr15_40627444	C15orf52	C15orf52:NM_207380:exon11:c.C1520A:p.P507H	0	1	not confirmed	NA
chr9_74667232	C9orf57	C9orf57:NM_001128618:exon5:c.C466A:p.H156N	0	1	confirmed	Heterozygous in 1 healthy control and homozygous reference in 1 case
chr9_74671095	C9orf57	C9orf57:NM_001128618:exon4:c.T241C:p.C81R	0	1	confirmed	Homozygous reference in only 1 available control
chr19_1037781	CNN2	CNN2:NM_201277:exon6:c.C695A:p.P232H	$1.00x10^{-04}$	2	not confirmed	NA
chr19_1037807	CNN2	CNN2:NM_201277:exon6:c.G721A:p.A241T	$3.16x10^{-05}$	1	not confirmed	NA
chr12_56334185	DGKA	DGKA:NM_001345:exon11:c.A886G:p.T296A	0.0036	1	confirmed	Heterozygous in 3 controls
chr12_56345838	DGKA	DGKA:NM_001345:exon19:c.G1607A:p.R536Q	$1.50x10^{-05}$	1	confirmed	1 case with memory complaint is heterozygous, other cases and controls homozygous ref
chr2_88890481	**EIF2AK3**	**EIF2AK3:NM_004836:exon5:c.A857G:p.N286S**	**$3.00x10^{-05}$**	**1**	**confirmed**	**Heterozygous in one healthy control**
chr2_88892838	**EIF2AK3**	**EIF2AK3:NM_004836:exon4:c.G719A:p.R240H**	**$8.00x10^{-04}$**	**1**	**confirmed**	**Heterozygous in one healthy control**
chr19_14863149	EMR2	EMR2:NM_001271052:exon13:c.G1606T:p.G536X	$8.00x10^{-04}$	1	confirmed	no controls or cases available
chr19_14866600	EMR2	EMR2:NM_001271052:exon12:c.C1282T:p.L428F	0.0011	1	confirmed	Heterozygous in all 3 controls
chr19_4793724	FEM1A	FEM1A:NM_018708:exon1:c.C1858A:p.L620M	0.0029	3	not confirmed	NA
chr12_42481670	GXYLT1	GXYLT1:NM_001099650:exon7:c.G1148A:p.C383Y	$3.00x10^{-04}$	2	not confirmed	NA
chr12_42481671	GXYLT1	GXYLT1:NM_001099650:exon7:c.T1147C:p.C383R	$3.00x10^{-04}$	2	not confirmed	NA
chr12_42499738	GXYLT1	GXYLT1:NM_001099650:exon4:c.A653G:p.E218G	0	1	not confirmed	NA

Supplementary Table 2. *Continued*

Location	Gene	Variant information	ExAC NFE	Number of families	Sanger sequencing WES cases	Sanger sequencing in related cases and controls
chr12_42499739	GXYLT1	GXYLT1:NM_001099650:exon4:c.G652A:p.E218K	0	1	not confirmed	NA
chr3_151163948	IGSF10	IGSF10:NM_178822:exon4:c.C3821T:p.T1274M	$7.49x10^{-05}$	1	confirmed	Homozygous reference in all 3 controls
chr3_151165241	IGSF10	IGSF10:NM_178822:exon4:c.C2528G:p.S843C	0.0011	1	confirmed	Homozygous reference in 2 other cases and 2 controls
chr3_151174852	IGSF10	IGSF10:NM_178822:exon2:c.A286G:p.I96V	$1.00x10^{-04}$	1	confirmed	Homozygous reference in 2 other cases and 2 controls
chr21_26976105	MRPL39	MRPL39:NM_080794:exon4:c.420+3A>G	0	1	confirmed	Homozygous reference in 2 other cases and 2 controls
chr21_26978806	MRPL39	MRPL39:NM_017446:exon2:c.T235G:p.F79V	$4.00x10^{-04}$	1	confirmed	heterozygous in 2 controls
chrX_64949483	MSN	MSN:NM_002444:exon4:c.G376A:p.A126T	0	2	not confirmed	NA
chrX_64949484	MSN	MSN:NM_002444:exon4:c.C377T:p.A126V	0	2	not confirmed	NA
chr19_8999443	MUC16	MUC16:NM_024690:exon56:c.A40732G:p.I13578V	$1.50x10^{-05}$	3	Excluded	NA
chr19_8999478	MUC16	MUC16:NM_024690:exon56:c.A40697G:p.Q13566R	0.0032	1	Excluded	NA
chr19_8999497	MUC16	MUC16:NM_024690:exon56:c.40674_40677del:p.K13558fs	$2.00x10^{-04}$	1	Excluded	NA
chr19_8999502	MUC16	MUC16:NM_024690:exon56:c.40672_40673insTCGG:p.K13558fs	$2.00x10^{-04}$	1	Excluded	NA
chr19_8999511	MUC16	MUC16:NM_024690:exon56:c.C40664T:p.P13555L	$2.00x10^{-04}$	1	Excluded	NA
chr19_8999512	MUC16	MUC16:NM_024690:exon56:c.C40663G:p.P13555A	$2.00x10^{-04}$	1	Excluded	NA
chr19_8999518	MUC16	MUC16:NM_024690:exon56:c.T40657C:p.Y13553H	$1.00x10^{-04}$	2	Excluded	NA
chr19_8999530	MUC16	MUC16:NM_024690:exon56:c.G40645A:p.A13549T	$9.21x10^{-05}$	1	Excluded	NA
chr19_8999538	MUC16	MUC16:NM_024690:exon56:c.A40637G:p.K13546R	$9.26x10^{-05}$	1	Excluded	NA
chr19_8999539	MUC16	MUC16:NM_024690:exon56:c.A40636G:p.K13546E	$6.17x10^{-05}$	1	Excluded	NA
chr19_8999550	MUC16	MUC16:NM_024690:exon56:c.G40625A:p.G13542E	$6.19x10^{-05}$	1	Excluded	NA
chr19_8999554	MUC16	MUC16:NM_024690:exon56:c.G40621C:p.D13541H	$4.66x10^{-05}$	1	Excluded	NA
chr19_9009325	MUC16	MUC16:NM_024690:exon40:c.T39148A:p.L13050M	$3.00x10^{-04}$	1	Excluded	NA

Supplementary Table 2. *Continued*

Location	Gene	Variant information	ExAC NFE	Number of families	Sanger sequencing WES cases	Sanger sequencing in related cases and controls
chr19_9009331	MUC16	MUC16:NM_024690:exon40:c.G39142A:p.G13048S	$3.00x10^{-04}$	1	Excluded	NA
chr19_9009637	MUC16	MUC16:NM_024690:exon39:c.A39089G:p.H13030R	0	2	Excluded	NA
chr11_1260217	MUC5B	MUC5B:NM_002458:exon26:c.C3414A:p.D1138E	0	4	Excluded	NA
chr11_1260219	MUC5B	MUC5B:NM_002458:exon26:c.C3416T:p.A1139V	0	4	Excluded	NA
chr11_1017933	MUC6	MUC6:NM_005961:exon31:c.T4868C:p.L1623S	0.0038	8	Excluded	NA
chr11_1017939	MUC6	MUC6:NM_005961:exon31:c.C4862G:p.T1621S	0.0021	8	Excluded	NA
chr17_48585959	MYCBPAP	MYCBPAP:NM_032133:exon1:c.G53T:p.W18L	$3.00x10^{-04}$	1	confirmed	no healthy controls available
chr17_48605632	MYCBPAP	MYCBPAP:NM_032133:exon16:c.2536+1G>A	$1.91x10^{-05}$	1	confirmed	heterozygous in 1 out 3 control
chr17_10298599	MYH8	MYH8:NM_002472:exon34:c.G4813A:p.D1605N	0.0028	1	confirmed	no healthy controls available
chr17_10304362	MYH8	MYH8:NM_002472:exon25:c.3254+1G>A	$3.00x10^{-04}$	1	confirmed	Heterozygous in one control and homozygous reference in 1 case and other controls
chr8_101721709	PABPC1	PABPC1:NM_002568:exon8:c.A1223T:p.Y408F	0.0046	1	not confirmed	NA
chr8_101730110	PABPC1	PABPC1:NM_002568:exon3:c.T394G:p.C132G	0.0026	1	not confirmed	NA
chr8_101730117	PABPC1	PABPC1:NM_002568:exon3:c.388-1G>A	0.0013	1	not confirmed	NA
chr13_25670801	PABPC3	PABPC3:NM_030979:exon1:c.465_466insG:p.I155fs	0	1	not confirmed	NA
chr13_25670803	PABPC3	PABPC3:NM_030979:exon1:c.A467G:p.K156R	0	1	not confirmed	NA
chr13_25670877	PABPC3	PABPC3:NM_030979:exon1:c.G541A:p.A181T	$9.00x10^{-04}$	7	not confirmed	NA
chr13_25671168	PABPC3	PABPC3:NM_030979:exon1:c.C832T:p.R278C	$1.00x10^{-04}$	2	not confirmed	NA
chr13_25671172	PABPC3	PABPC3:NM_030979:exon1:c.C836A:p.T279K	$1.00x10^{-04}$	2	not confirmed	NA
chr13_25671271	PABPC3	PABPC3:NM_030979:exon1:c.A935G:p.K312R	0	4	not confirmed	NA
chr13_25671272	PABPC3	PABPC3:NM_030979:exon1:c.937delG:p.A313fs	0	4	not confirmed	NA
chr13_25671274	PABPC3	PABPC3:NM_030979:exon1:c.C938T:p.A313V	0	5	not confirmed	NA

Supplementary Table 2. *Continued*

Location	Gene	Variant information	ExAC NFE	Number of families	Sanger sequencing WES cases	Sanger sequencing in related cases and controls
chr13_25671292	PABPC3	PABPC3:NM_030979:exon1:c.C956T:p.T319I	0	4	not confirmed	NA
chr13_25671672	PABPC3	PABPC3:NM_030979:exon1:c.A1336G:p.S446G	0.0047	1	not confirmed	NA
chr11_67166306	PPP1CA	PPP1CA:NM_206873:exon5:c.T637A:p.F213I	0	3	not confirmed	NA
chr11_67166312	PPP1CA	PPP1CA:NM_206873:exon5:c.630_631insA:p.Y211fs	0	2	not confirmed	NA
chr14_94731284	PPP4R4	PPP4R4:NM_058237:exon20:c.C2197T:p.R733C	0.0028	1	confirmed	Heterozygous in 2 controls and homozygous reference in 1 control; no additional cases
chr14_94732219	PPP4R4	PPP4R4:NM_058237:exon22:c.T2373G:p.C791W	$2.00x10^{-04}$	1	confirmed	Heterozygous in one control; no additional cases
chr9_33797928	PRSS3	PRSS3:NM_001197098:exon3:c.281_282insCC:p.R94fs	0	3	not confirmed	NA
chr9_33797930	PRSS3	PRSS3:NM_001197098:exon3:c.284_285del:p.D95fs	0	4	confirmed	Heterozygous in more controls incl. pathological confirmed non-demented controls
chr9_33797951	PRSS3	PRSS3:NM_001197098:exon3:c.A304C:p.M102L	$1.00x10^{-04}$	4	not confirmed	NA
chr9_33797987	PRSS3	PRSS3:NM_001197098:exon3:c.G340T:p.A114S	0.0015	6	not confirmed	NA
chr9_33797999	PRSS3	PRSS3:NM_001197098:exon3:c.A352G:p.T118A	0.0012	6	not confirmed	NA
chr7_56087291	PSPH	PSPH:NM_004577:exon5:c.275+1G>-	0.0014	2	not confirmed	NA
chr20_42199704	SGK2	SGK2:NM_016276:exon8:c.690+4GGTGTGT>G	0	1	confirmed	Heterozygous in controls and pathological confirmed non-demented. Known snp nearby with almost same mutation
chr20_42199704	SGK2	SGK2:NM_016276:exon8:c.690+4GGT>G	0	1	confirmed	Heterozygous in control and pathological confirmed non-demented. Known snp nearby with almost same mutation
chr22_51117800	SHANK3	SHANK3:NM_033517:exon7:c.G829A:p.G277R	0	3	not confirmed	NA
chrX_118603958	SLC25A5	SLC25A5:NM_001152:exon2:c.G446A:p.G149E	$4.00x10^{-04}$	2	not confirmed	NA
chrX_118603961	SLC25A5	SLC25A5:NM_001152:exon2:c.450delT:p.A150fs	$4.00x10^{-04}$	2	not confirmed	NA
chrX_118603984	SLC25A5	SLC25A5:NM_001152:exon2:c.G472A:p.G158S	$3.00x10^{-04}$	1	not confirmed	NA

Supplementary Table 2. *Continued*

Location	Gene	Variant information	ExAC NFE	Number of families	Sanger sequencing WES cases	Sanger sequencing in related cases and controls
chrX_118604006	SLC25A5	SLC25A5:NM_001152:exon2:c.A494G:p.Y165C	$2.00x10^{-04}$	1	not confirmed	NA
chr4_103822492	SLC9B1	SLC9B1:NM_139173:exon12:c.1333-3C>T	0.0028	2	not confirmed	NA
chr5_159839530	SLU7	SLU7:NM_006425:exon6:c.571-4->T	0	1	confirmed	Heterozygous in controls (incl pathological non demented)
chr5_159839530	SLU7	SLU7:NM_006425:exon6:c.571-4->TTTT	0	1	confirmed	Heterozygous in controls (incl pathological non demented)
chr5_159839530	SLU7	SLU7:NM_006425:exon6:c.571-4->TTTTT	0	1	confirmed	Heterozygous in controls (incl pathological non demented)
chr12_11286746	TAS2R30	TAS2R30:NM_001097643:exon1:c.T98C:p.I33T	$6.00x10^{-04}$	3	not confirmed	NA
chr12_11244067	TAS2R43	TAS2R43:NM_176884:exon1:c.761_762insAA:p.S254fs	0.0015	4	not confirmed	NA
chr12_11244091	TAS2R43	TAS2R43:NM_176884:exon1:c.G738A:p.M246I	0.0042	4	not confirmed	NA
chr12_11244096	TAS2R43	TAS2R43:NM_176884:exon1:c.A733G:p.I245V	0.0044	4	not confirmed	NA
chr2_179452395	TTN	TTN:NM_003319:exon134:c.A36446C:p.D12149A	$3.02x10^{-05}$	1	confirmed	Heterozygous in one case, homozygous reference in two cases and 1 control
chr2_179462367	TTN	TTN:NM_003319:exon122:c.A30247G:p.M10083V	$6.00x10^{-04}$	1	confirmed	Heterozygous in one case and one healthy control, other controls homozygous reference
chr2_179478597	TTN	TTN:NM_003319:exon91:c.G22218T:p.W7406C	$6.00x10^{-04}$	1	confirmed	Homozygous reference in 4 controls; no additional cases
chr2_179643821	TTN	TTN:NM_003319:exon23:c.C3850T:p.R1284C	$1.51x10^{-05}$	1	confirmed	No cases or controls available
chr17_5036823	USP6	USP6:NM_004505:exon6:c.T362C:p.L121S	0.0028	4	not confirmed	NA
chr19_38375738	WDR87	WDR87:NM_031951:exon6:c.A8456G:p.Q2819R	0.0039	1	confirmed	Heterozygous in one case heterozygous and two controls; One other case is homozygous reference
chr19_38384964	WDR87	WDR87:NM_031951:exon4:c.A1262C:p.Q421P	0	1	confirmed	Homozygous reference in 3 controls; no additional cases
chr4_338197	ZNF141	ZNF141:NM_003441:exon3:c.204_205insGA:p.H68fs	$5.00x10^{-04}$	4	not confirmed	NA

Supplementary Table 2. *Continued*

Location	Gene	Variant information	ExAC NFE	Number of families	Sanger sequencing WES cases	Sanger sequencing in related cases and controls
chr4_338200	ZNF141	ZNF141:NM_003441:exon3:c.G207C:p.K69N	$5.00x10^{-04}$	4	not confirmed	NA
chr4_338201	ZNF141	ZNF141:NM_003441:exon3:c.209_210del:p.I70fs	$5.00x10^{-04}$	4	not confirmed	NA
chr4_338205	ZNF141	ZNF141:NM_003441:exon3:c.T212C:p.V71A	$5.00x10^{-04}$	4	not confirmed	NA
chr2_71625864	ZNF638	ZNF638:NM_001014972:exon12:c.C2462A:p.T821K	0.0035	2	confirmed	Family 1: heterozygous in one case and control; Seven controls were homozygous reference Family 8: no controls available

NA, not applicable; ExAC NFE, Exome Aggregation Consortium Non Finnish European.

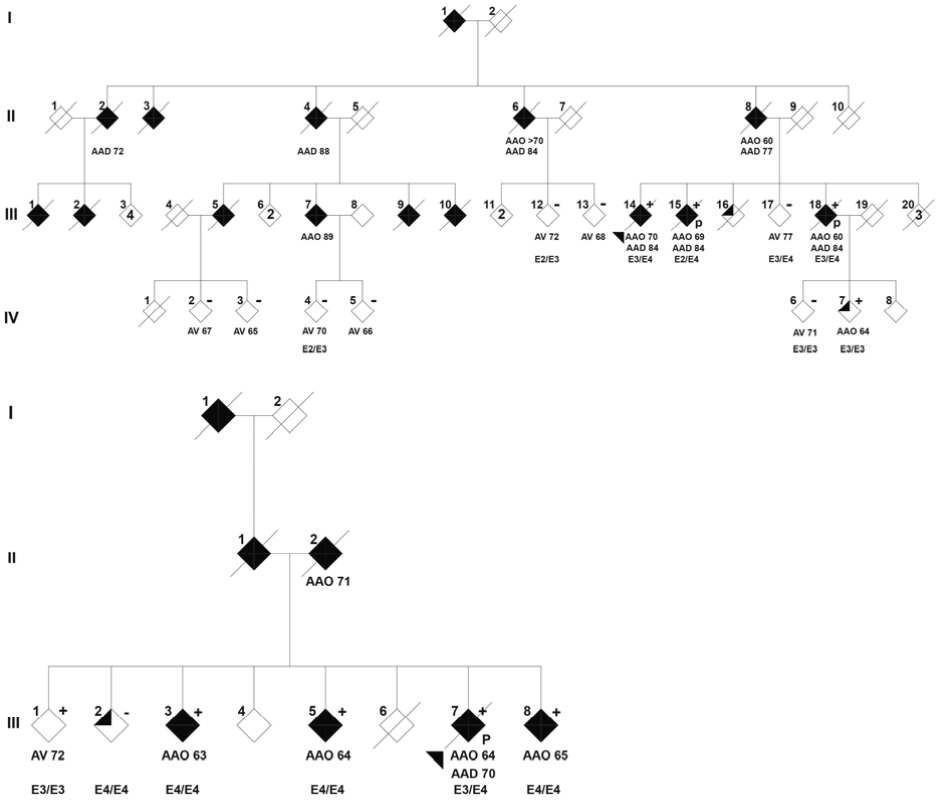
Supplementary Table 3. *EIF2AK3* variants in the Dutch exome sequencing data

Position	cDNA base Change	Amino acid changes	Exon	dbsnp142	Function	CADD	ExAC NFE	MAF	*p*-value	Odd ratio
88857400	3205A>G	I1069V	17	.	Missense	0.21	0	0.0003	0.45	0.21 (0 - 12.14)
88870404	2973G>A	M991I	14	.	Missense	33.00	0	0.0003	0.50	0.23 (0 - 16.46)
88870458	2919C>G	T973T	14	rs374698464	Synonymous	8.44	0	0.0003	0.50	0.24 (0 - 16.48)
88874264	2737G>A	V913M	13	.	Missense	10.61	$7.50x10^{-05}$	0.0004	0.43	0.19 (0 - 11.33)
88874534	2467A>G	K823E	13	rs374540128	Missense	7.34	$4.50x10^{-05}$	0.0003	0.14	25.24 (0.36 - 1768.75)
88874854	2147C>T	P716L	13	rs55861585	Missense	12.14	0.0006	0.0003	0.19	15.1 (0.26 - 892.59)
88874891	2110G>T	A704S	13	rs1805165	Missense	15.98	0.7193	0.2870	0.26	1.1 (0.93 - 1.29)
88879131	1791A>G	Q597Q	11	rs1805164	Synonymous	0.23	0.3024	0.3240	0.83	0.98 (0.83 - 1.16)
88882924	G>A	NA	-	.	Intronic	7.84	$1.50x10^{-05}$	0.0003	0.19	15.01 (0.25 - 887.11)
88882942	1763+6A>T	1763+6A>T	11	rs6750998	Splicing	10.84	0.2912	0.2730	0.70	0.97 (0.82 - 1.14)
88882955	1756A>T	I586L	10	rs75385605	Missense	7.25	$7.50x10^{-05}$	0.0003	0.13	25.73 (0.37 - 1803.1)
88882992	1719T>C	N573N	10	rs137927384	Synonymous	7.16	0.0003	0.0003	0.19	15.6 (0.26 - 922.22)
88883014	1697A>T	D566V	10	rs55791823	Missense	24.50	0.0043	0.0100	0.29	1.54 (0.7 - 3.43)
88885505	1504G>A	D502N	9	rs141901506	Missense	18.36	0.0008	0.0020	0.11	0.22 (0.03 - 1.38)
88888294	1291T>G	W431G	7	.	Missense	29.00	0	0.0003	0.44	0.2 (0 - 11.84)
88890481[a]	857A>G	N286S	5	rs150474217	Missense	0.002	$3.0x10^{-5}$	NA	NA	NA

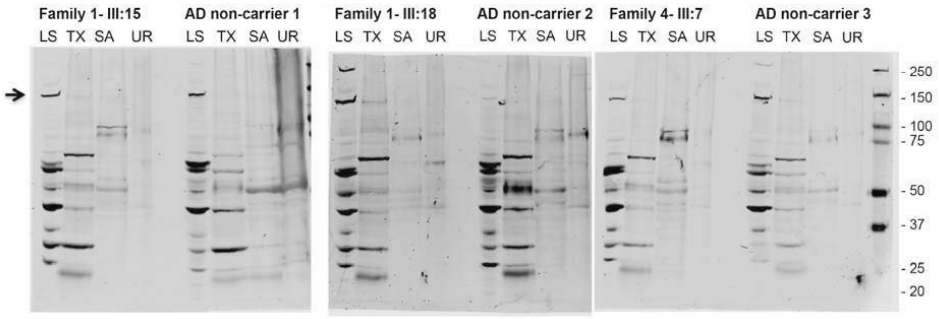
Supplementary Table 3. *Continued*

Position	cDNA base Change	Amino acid changes	Exon	dbsnp142	Function	CADD	ExAC NFE	MAF	*p*-value	Odd ratio
88890552	786C>A	G262G	5	rs375371096	Synonymous	8.31	$1{,}50x10^{-05}$	0.0003	0.14	25.51 (0.36 - 1787.83)
88892838	**719G>A**	**R240H**	**4**	**rs147458427**	**Missense**	**31.00**	**0,0008**	**0.0030**	**0.04**	**4.22 (1.06 - 16.80)**
88892892	665G>A	R222H	4	rs144117387	Missense	24.20	0	0.0003	0.18	15.86 (0.27 - 937.64)
88895012	608A>G	Y203C	3	.	Missense	23.60	$3{,}01x10^{-05}$	0.0003	0.49	0.23 (0 - 15.91)
88895016	604A>G	T202A	3	.	Missense	25.00	0	0.0003	0.14	24.68 (0.35 - 1729.56)
88895064	556T>C	Y186H	3	rs200991366	Missense	19.86	0,0002	0.0007	0.68	1.85 (0.1 - 33.15)
88913257	423C>A	S141R	2	.	Missense	31.00	0	0.0003	0.19	14.94 (0.25 - 883.36)
88913273	407C>G	S136C	2	rs867529	Missense	18.27	0,2808	0.3020	0.49	1.06 (0.9 - 1.25)

Variants in *EIF2AK3* (NM 004836) on chromosome 2 which are found in the Dutch exome sequencing data. The variant in bold is also genotyped in an additional cohort from the Rotterdam study. a, variant is only found in family NLAD4. CADD, Combined Annotation Dependent Depletion score; ExAC NFE, Exome Aggregation Consortium Non Finnish European; MAF, Minor Allele Frequency in the Dutch exome dataset; NA, not applicable. The p-value and odds ratio of the single variant analysis in the Dutch exome cohort. Only p.R240H has a p-value below 0.05, but is not significant when corrected for Bonferroni.



Supplementary Figure 1. Pedigrees of families with *EIF2AK3* variants. The upper pedigree shows the family (NLAD 1) of patients with Alzheimer's disease (AD) with *EIF2AK3* p.R240H variant, and the lower pedigree the family (NLAD 4) with the *EIF2AK3* p.N285S variant. Filled symbols are affected individuals, and a quarter-filled symbols are individuals with subjective memory complaints. Symbols with a diagonal line represent deceased individuals. The proband is indicated with triangle. Numbers in the symbols indicate the number of individuals. The individuals III:14, III:15 and III:18 from NLAD 1 and individuals III:3 and III:4 from NLAD 4 were selected for whole exome sequencing. Plus sign indicates that the individual carry the variant, and minus sign indicates that the variant is absent. *APOE* status is available for subset of the included individuals. AAO, age at onset; AAD: Age at death; AV, Age at last visit; P, Pathological confirmed AD.



Supplementary Figure 2. Immunoblot analysis of PERK in *EIF2AK3* carriers and AD non-*EIF2AK3* carriers. Proteins were extracted from the frontal cortex of *EIF2AK3* carriers and non-EIF2AK3 carriers with Alzheimer's disease. Antibody against pPERK showed a band of ~140 kDa in low salt (LS), which is indicated with the arrow. We found no differences in solubility of pPERK between *EIF2AK3* carriers and non-*EIF2AK3* carriers. Also, no difference in banding pattern was seen between *EIF2AK3* carriers and non-*EIF2AK3* carriers. Low salt (LS), TritonTM X-100 (TX), sarkosyl (SA) and urea (UR).

CHAPTER 3

3

Genetic heterogeneity in frontotemporal lobar degeneration

CHAPTER 3.1

3.1

Three *VCP* mutations in patients with frontotemporal dementia

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Abstract

Valosin-containing protein (VCP) is involved in multiple cellular activities. Mutations in *VCP* lead to heterogeneous clinical presentations including inclusion body myopathy with Paget's disease of the bone, frontotemporal dementia and amyotrophic lateral sclerosis, even in patients carrying the same mutation. We screened a cohort of 48 patients with familial frontotemporal dementia (FTD) negative for *MAPT*, *GRN* and *C9orf72* mutations for other known FTD genes by using whole exome sequencing. In addition, we carried out targeted sequencing of a cohort of 37 patients with frontotemporal lobar degeneration with transactive response DNA-binding protein 43 (TDP-43) subtype from the Netherlands Brain bank. Two novel (p.Thr262Ser and p.Arg159Ser) and one reported (p.Met158Val) *VCP* mutations in three patients with a clinical diagnosis of FTD were identified, and were absence in population-match controls. All three patients presented with behavioral changes, with additional semantic deficits in one. No signs of Paget or muscle disease were observed. Pathological examination of the patient with *VCP* p.Arg159Ser mutation showed numerous TDP-43 immunoreactive (IR) neuronal intranuclear inclusions (NII) and dystrophic neurites (DN), while a lower number of NII and DN were observed in the patient with the *VCP* p.Thr262Ser mutation. Pathological findings of both patients were consistent with FTLD-TDP subtype D. Furthermore, only rare VCP-IR NII was observed in both cases. Our study expands the clinical heterogeneity of *VCP* mutations carriers, and indicates that other additional factors, such as genetic modifiers, may determine the clinical phenotype.

Introduction

Valosin-containing protein (*VCP*), also known as p97, is a member of ATPase Associated with diverse cellular Activities protein family.[1, 2] VCP is composed of an N-terminal domain (CDC48) involved in ubiquitin binding, two D1-and D2 domains that bind and hydrolyze ATP, and a flexible C-terminal region.[1, 3] These structures can be assembled into a homohexamer.[3, 4] VCP is involved in multiple cellular processes including protein degradation via ubiquitin proteasome system,[5] cell division, nuclear envelope formation,[6] endoplasmic reticulum associated protein degradation (ERAD),[7] aggresome formation,[8] Golgi apparatus assembly, autophagy, mitophagy, endosomal trafficking, cell cycle, and DNA repair.[9]

The classical phenotype of mutations in *VCP* is inclusion body myopathy with Paget's disease of the bone and frontotemporal dementia (IBMPFD) with an autosomal dominant inheritance.[10] IBMPFD is characterized by proximal and distal muscle weakness resembling a limb-girdle dystrophy syndrome, Paget disease of bone and frontotemporal dementia (FTD).[11] Currently, different mutations in domains linker 1, D1 and predominantly CDC48 are reported to this multisystem disease.[12] The phenotype of these mutations can be variable, even within the same family: patients could solely present with myopathy, FTD or amyotrophic lateral sclerosis (ALS).[12-15] Myopathy is the most common presenting features of this disease, and is found in about 91% of the patients, while an FTD phenotype was observed in approximately 30% of patients with *VCP* mutations.[16] Pathologically, transactive response DNA binding protein of 43 kDa (TDP-43) and p62 immunoreactive (IR) short dystrophic neurites (DN) with neuronal intranuclear inclusions (NII), classified as FTLD-TDP type D, are characteristic for *VCP* mutations.[17, 18]

In this study, we sequenced a cohort of 48 patients using whole exome sequencing (WES) and analyzed 37 pathological confirmed FTLD-TDP cases using targeted sequencing, leading to the identification of three mutations in *VCP* (including two novel) in three unrelated Dutch patients presenting with FTD. Here, we here describe the clinical and available pathological characteristics of these mutation carriers.

Methods

Subjects

The Dutch FTD cohort included patients with a clinical diagnosis of FTD, ascertained as part of an on-going genetic-epidemiological study in the Netherlands since 1994.[19] Detailed clinical information of the patients was obtained by interviewing relatives and reviewing medical records from hospitals or nursing homes. Patients underwent a neurological examination and neuropsychological assessment when possible.

Neuroimaging including computed tomography (CT), magnetic resonance imaging (MRI) and single-photo emission computed tomography (SPECT) was also reviewed when available. The diagnosis of behavioural variant of FTD (bvFTD) was established according to the international consensus criteria by Rascovsky,[20] and ALS was diagnosed when patients met El Escorial criteria.[21] Pathological diagnosis of frontotemporal lobar degeneration (FTLD) is confirmed by the pathologist of the Netherlands Brain Bank.

In the Dutch FTD cohort, we selected all patients with FTD and with a positive family history for dementia or ALS, but without mutations in *MAPT*, *GRN* and repeat expansion in *c9orf72* (*n*=48). The mean age at onset was 59.0 ± 9.4 years (range 36.2-73.9), and the mean age at death was 67.3 ± 9.9 years (range 42.5-82.7). Pathological diagnosis of FTLD was confirmed in 13 patients (FTLD-TDP in 12 and 1 FTLD-Tau), and no autopsy was performed in the remaining 35 patients. Additionally, a cohort of 37 patients with FTLD-TDP without mutations in *GRN* and repeat expansion in *c9orf72* from the Netherlands Brain Bank was selected for targeted sequencing (data of family history were incomplete). The mean age at onset of the latter cohort was 59.9 ± 7.4 years (range 45.2-80.5). The mean age at death was 68.3 ± 6.4 (range 54.5-87.4).

DNA was obtained from blood in patients selected for WES and was extracted from cerebellar brain tissue of patients selected for targeted sequencing. The study is approved by the Medical Ethical Committee of the Erasmus Medical Centre and the Netherlands Brain Bank, and informed consent is obtained from all participants.

Genetic and bioinformatic analysis

Whole exome sequencing was performed using Nimblegen v2 Seqcap EZ Exome Kit (Roche) for exome capturing. DNA was prepared with the Illumina TruSeq Paired-End Library Preparation Kit according to manufacturer's instructions, and sequenced on a HiSeq 2000 (Illumina, San Diego, CA). The data was generated at the Human Genomics Facility (HuGeF; www.glimdna.org) at Erasmus MC according to an in-house pipeline.

Targeted sequencing was performed using customized exon capture kit. A list of known neurodegenerative disease genes was captured using custom-designed targeted panel created by SureDesign (Agilent Technologies, Santa Clara, CA). DNA library was prepared by NEBNext Ultra DNA Library Prep Kit for Illumina according to manufacturer's instructions, and paired-end sequencing was performed using Illumina HiSeq 4000 (Illumina, San Diego, CA). Sequences were processed in-house using Mayo Clinic's DNA analysis pipeline Genome GPS v4.0.

Alignment of the sequencing reads to the human reference genome GRCh37 was performed using Burrows-Wheeler Aligner.[22] After alignment, duplicate reads were marked and removed using Picard (v1.119).[23] HaplotypeCaller from Genome Analysis Toolkit (GATK) was used for variant calling, and quality control using variant quality score

recalibration and hard filters according to GATK best practice. Functional annotations of variant sites were performed using ANNOVAR.[24] Combined Annotation Dependent Depletion (CADD) score (v1.3) was used to predict the pathogenicity of the variants.[25] In both sequencing data, we focused on disease-causing genes in AD and FTD (*APP*, *PSEN1*, *PSEN2*, *MAPT*, *GRN*, *TARDBP*, *VCP*, *SQSTM1*, *CHMP2B*, *CHCHD10*, *FUS*, *CSF1R*, *TREM2*, *UBQLN2*, *SOD1*, *OPTN* and *TBK1*). Non-synonymous or protein truncating variants (including nonsense or frameshift INDELS) with minor allele frequency of <0.01% from the genome Aggregation Database (gnomAD) were retained.26 Additionally, we used the sequencing data from Genome of the Netherlands (GoNL) and exome data of non-demented individuals from the Rotterdam study (*n*=2101) to filter out polymorphism.[27, 28]

All candidate variants were validated by Sanger sequencing, and sequenced on an ABI3730xl genetic analyzer (Applied Biosystems, CA, USA).

Histology and immunohistochemistry

Brain autopsy was performed in the p.Thr262Ser and p.Arg159Ser *VCP* mutation carriers by the Netherlands Brain Bank. Immunohistochemical staining was performed using the following antibodies: Hyperphosphorylated tau (AT8, 1:40, Innogenetics), amyloid-β protein (1:100, DAKO), p62 (1:200, BD Biosciences Pharmingen), phospho (p) TDP-43 (1:100, Biotech) and VCP (ab11433-50; 1:500, Abcam). The pathological diagnosis of FTLD was made by neuropathologist (A.J.M.R.). TDP-43 pathology was classified in subtypes according to the algorithm described by Mackenzie et al.[18]

Results

Genetic analysis

In the WES cohort, we found three rare nonsynonymous variants after filtering: two in *VCP* (p.Thr262Ser and p.Met158Val), and one in *TARDBP* (p.Ile383Val). In the targeted sequencing data, we found one additional variant in *VCP* (p.Arg159Ser) and one variant in *APP* (p.G119R). Sanger sequencing confirmed all variants. The variant in *APP* gene was most likely not pathogenic due to its location (exon 4). The variant in *TARDBP* had been reported previously.[29] All three variants in *VCP* were unknown in gnomAD, GoNL and exome data from the Rotterdam Study, and two of these (p.Thr262Ser and p.Arg159Ser) have not been reported previously (Table 1).

Clinical features of VCP mutations carriers

Patient 1 (p.Met158Val) - At 41 years old, this patient presented with reduced empathy, loss of interest in grooming, reduced verbal output, and increased aggressiveness.

Table 1. Summary of clinical characteristics of *VCP* mutation carriers

Amino acid change	cDNA base change	Exon	CADD	Protein domain	Age at onset	Initial presenting symptoms	Language problems[a]	Clinical diagnosis	Reported phenotype at same codon [12]
p.Met158Val	c.472A>G	5	22.8	CDC48	41	Behavioral changes	semantic deficits	svFTD	ALS
p.Arg159Ser	c.475C>A	5	26.1	CDC48	56	Behavioral changes	no	bvFTD	FTD (with PDB), ALS (with PDB), IBM, LGMD-like without PDB, HSP with PDB, IBMPFD1, peripheral neuropathy
p.Thr262Ser	c.785C>G	7	22.8	D1	60	Behavioral changes	no	bvFTD	IBM, PDB

[a] language problems early in the disease process; CADD, Combined Annotation Dependent Depletion; svFTD, semantic variant frontotemporal dementia; bvFTD, behavioral variant frontotemporal dementia; ALS, Amyotrophic Lateral Sclerosis; PDB, Paget disease of the bone; IBM, Inclusion Body Myopathy; LGMD, Limb-Girdle Muscular Dystrophy; HSP, Hereditary Spastic Paraplegia; IBMPFD, Inclusion Body Myopathy with Paget's disease of the bone and frontotemporal dementia.

Prominent difficulties with finding words and naming objects were noticed early in the disease process. He was also obsessive in collecting and disassembling devices, and was ticking on couches. Neurological examination one year after disease onset showed word finding problems and short-term memory impairment. Fasciculations or muscle weakness were absent. Routine blood tests showed a normal calcium, phosphate and alkaline phosphatase. Neuropsychological assessment revealed lack of disease insight, semantic deficits, executive dysfunction, and to a lesser extent impairment of memory and visual constructive function. MRI showed cortical atrophy and moderate bilateral temporal atrophy. The clinical diagnosis was bvFTD with pronounced semantic deficits. Patient died at the age of 46. No autopsy was performed. Family history showed no dementia in his both parents at age of 64, but dementia with behavioral changes in his grandfather on father's side at the age of 55 (1A), and dementia in his 79 year-old grandmother. Both grandparents on mother's side developed dementia at old age.

Patient 2 (p.Arg159Ser) – At 56 years old, this female patient developed personality changes and lost interest in her hobbies. She became egoistic and aggressive towards her husband, and she had transiently suicidal thoughts. There were no memory complaints and her orientation was normal. Her spontaneous speech diminished, and finally she became mute. CT-scan at 59 years old showed enlarged ventricles with cortical atrophy. The patient died at the age of 62 due to cachexia. Brain autopsy was performed. Family history revealed that her mother developed dementia at the age of 58 years (Fig. 1B).

Patient 3 (p.Thr262Ser) – At age 60, this female patient presented with reduced empathy, childish behavior, withdrawal from social contact and loss of interest in grooming (Table 1). She spent money on charities and gambling, and was drinking excessive amounts of alcohol. She developed memory problems and became disoriented. She also lost her skills in playing violin at professional level. Apathy, wandering and reduced verbal output were observed at the age of 64 years. She was admitted in the hospital with epileptic seizures, which was treated with anti-epileptics. Neurological examination five years after disease onset revealed reduced verbal output with echolalia. Signs of myopathy or bone disease, muscle weakness or fasciculations were absent. At neuropsychological testing, she had impairment in fluency, executive tasks and semantic paraphasias. Routine blood and cerebrospinal fluid examination revealed no abnormalities, with normal alkaline phosphatase and phosphate. CT scan showed atrophy of the frontal lobes and SPECT showed frontal hypoperfusion. The clinical diagnosis bvFTD was established. In the end stage of her disease, epileptic seizures increased in frequency, and she died at the age of 67. Brain autopsy was performed. Family history revealed that her mother presented with hyperorality, roaming and disorientation suggestive for the clinical diagnosis of bvFTD; she died at 62 years of age (Fig. 1C).

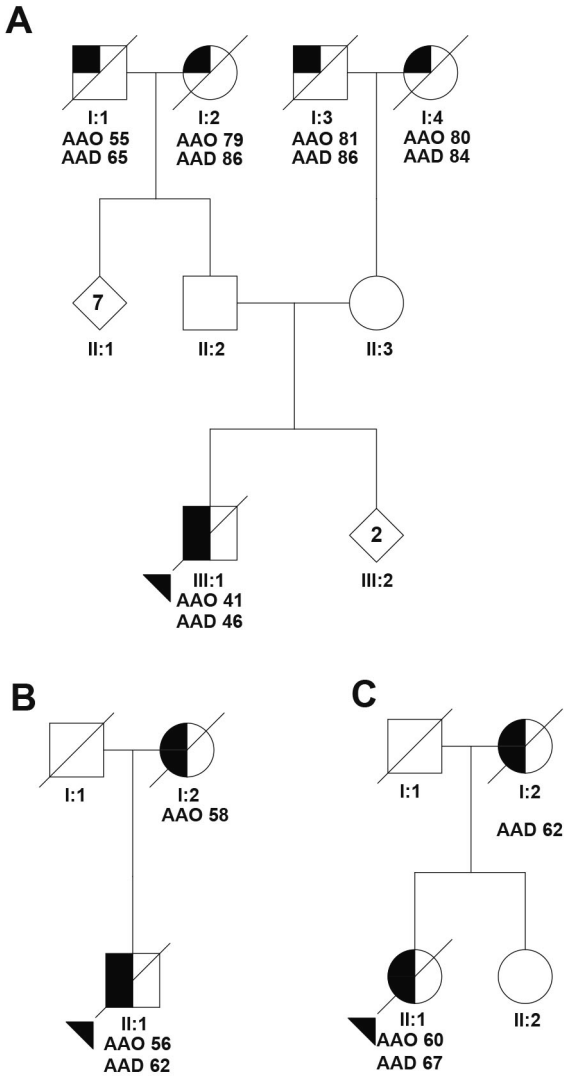

Figure 1. Pedigree of the VCP mutation carriers. Pedigrees show family members of p.Met158Val mutation carrier **(A)**, the p.Arg159Ser mutation carrier **(B)**, and the p.Thr262Ser mutation carrier **(C)**. Symbols with a diagonal line represent deceased individuals. The proband is indicated with the triangle. Numbers in the symbols indicate the number of individuals. Half-filled symbols represent individuals with Frontotemporal dementia and a quarter-filled symbols represent individuals with uncharacterized dementia. AAO, Age at onset; AAD, Age at death.

Neuropathological findings of p.Arg159Ser and p.Thr262Ser carriers

Macroscopically, prominent frontal atrophy was observed in both cases. Histological examination showed neuronal loss with gliosis in upper three neocortical layers, cornu amonnis (CA) 1, subiculum and parahippocampus, and in the insula and thalamus. Staining with pTDP-43 and p62 antibodies showed moderate to numerous NII and

short DN in all layers of the frontal and temporal cortex, and to a lesser extent in the parietal cortex (2A-2E), but none were detected in the dentate gyrus, CA regions of the hippocampus and occipital cortex. Abundant DN and NII were found in the neocortex of p.Arg159Ser case (2C and 2D), while much lesser DN and NII were observed in p.Thr262Ser (2A and 2B). Overall, round or lentiform-shaped NII of variable size and DN were found in the upper cortical layers, and less frequently in the deeper layers. A few TDP-43-IR neuronal cytoplasmic inclusions (NCI) were also found in the cortical regions of both cases, and were sporadically found in the medulla oblongata and spinal cord of p.Thr262Ser case. Both cases can be classified as FTLD-TDP type D.[18] VCP immunostaining revealed low numbers of NII scattered in the upper layer of the cortical regions (Figure 2F), but no NCI were found. Amyloid-β immunostaining showed a few senile plaques and sporadically neuritic plaques in p.Arg159Ser carrier corresponding with Thal 2, Braak 2 and Consortium to Establish a Registry for AD (CERAD) 0.[30] In p.Thr262Ser carrier, only a few hyperphosphorylated tau depositions without plaques were observed corresponding with Braak 1.

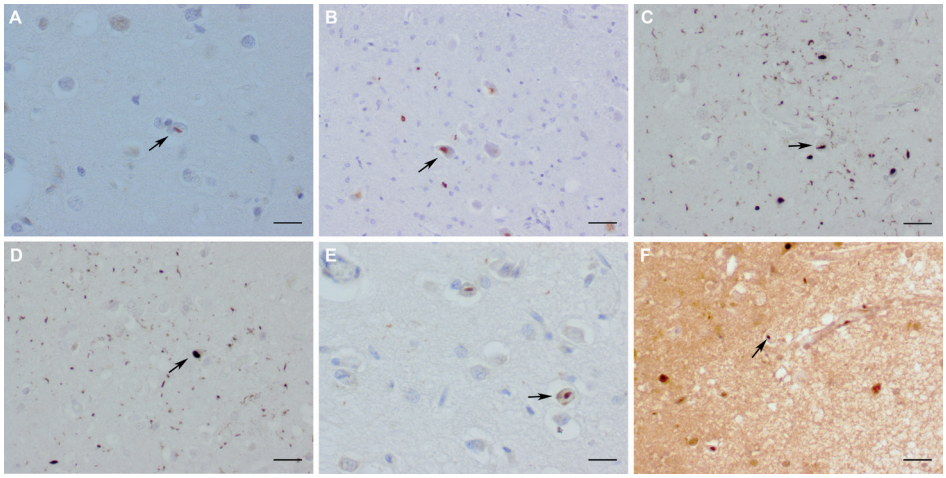

Figure 2. Immunohistochemistry of the brains from two VCP mutations carriers. Lentiform neuronal intranuclear inclusions (NII) positive for transactive response DNA binding protein of 43 kDa (TDP-43) immunohistochemical staining were observed in frontal cortex of p.Thr262Ser mutation carrier **(A)**. Some rare TDP-43 positive NII were also found in the brainstem of this case **(B)**. In contrast, numerous TDP-43 immunoreactive NII and dystrophic neurites (DN) were observed in all layers of the frontal **(C)** and temporal cortices **(D)** of p.Arg159Ser mutation carrier. The NII were also immunoreactive for p62 **(E)**. Staining of VCP revealed rare NII **(F)**. The arrows indicate the pathological findings. Scale bar is 20 µm.

Discussion

In this study, we identified two novel and one reported mutation in the *VCP* gene in patients with bvFTD, and we provided neuropathological features of the two novel mutations (p.Thr262Ser and p.Arg159Ser) carriers. The initial clinical presentation of

behavioral problems (including semantic deficits in one) without any signs of motor neuron disease or myopathy indicated the clinical diagnosis of bvFTD. Neuropathological findings in two patients showed numerous TDP43-IR NII and short DN compatible with FTLD-TDP type D and rare VCP-IR NII.

The pathogenicity of the three *VCP* mutations is supported by the fact that mutations at the same codon have been reported previously (Table 1).[13, 17, 31] Furthermore, the mutations in the present study are not present in population-matched controls, and were identified in the CDC48 and D1 domain, wherein most of the pathogenic *VCP* mutations have been reported.[12] The mutation p.Met158Val has previously been identified in a patient with sporadic ALS,[13] but it has not been reported in FTD before. The pathogenicity of this mutation is supported by increased translocation of TDP-43 from the nucleus to cytoplasm compared to those transfected with wild-type *VCP* in transfected SH-SY5Y cells and HEK293T cells.[13] Mutations in codon 159 and 262 have been reported in patients with variable phenotypes including, FTD, ALS, IBMPFD.[17, 31-33] The pathological findings of FTLD-TDP type D in our mutations carriers is consistent with previously reported findings of *VCP* mutation carriers.[17, 31]

The clinical presentation of pure FTD without any signs of Paget disease of the bone or myopathy in all three patients is in contrast to the classical phenotype of IBMPFD associated with mutations in *VCP*.[10] Distinct phenotypes including pure FTD or ALS have been described in other mutations at the same codons,[13, 31, 32] suggesting that additional genetic or environmental factors may determine the clinical phenotype. Another possibility might be a preclinical state of IBM or Paget disease, however, normal values of alkaline phosphatase in two patients made Paget disease unlikely. Furthermore, no muscle weakness or fasciculations were reported in neurological examinations ruling out IBM or ALS. The variation in age at onset and the unaffected parents of the proband in pedigree A also supported the presence of possible genetic modifiers in *VCP* mutations carriers.

The presence of VCP-IR NII in both cases indicated the involvement of mutant *VCP* in protein degradation. The neuropathological findings of numerous TDP43-IR NII and DN in both cases were similar to previously reported *VCP* mutations at the same codons.[17, 31] However, the distribution and the severity of the neuronal inclusions and DN differed between the two cases, with a lower number of NII and DN in the p.Thr262Ser carrier. These differences were unlikely to be related to the disease duration as the disease duration was shorter in the p.Arg159Ser carrier. A possible explanation is that the different location of the mutations may explain the extent of the pathology. More studies are needed to elucidate the disease mechanism of *VCP* mutations.

The frequency of 3.5% of *VCP* mutations could not be compared to other studies, as we screened a highly biased cohort of those patients without any other known mutations.

Our findings indicated that it is worthwhile to screen for *VCP* mutations, especially after excluding the most common mutations for FTD. In our Dutch FTD cohort, the frequency of *VCP* mutations was 4.2% in those with a positive family history. A limitation of the present study is that clinical information of the latest stages of the disease is unavailable. Patients were deceased many years ago, and were not followed until dead. At last, co-segregation analysis in families could not be performed due to the lack of DNA samples from relatives.

In conclusion, we report three *VCP* mutations, of which two novel mutations, in three Dutch patients with bvFTD. Pathological findings and previously reported mutations at the same codon support the pathogenicity of these mutations. Our findings expand the genetic and clinical spectrum of *VCP* mutations, and further underline the clinical heterogeneity. Future studies are warranted to investigate the involvement of genetic modifiers in the disease process of *VCP* carriers.

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CHAPTER 3.2

3.2

Novel *TUBA4A* variant is associated with familial frontotemporal dementia

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In preparation

Abstract

Tubulin alpha 4a (*TUBA4A*) encodes for α-tubulin that is an important component for microtubules assembly. Variants in *TUBA4A* have been suggested to be a rare genetic cause of amyotrophic lateral sclerosis, and have also been found in a minority of frontotemporal dementia (FTD) patients without supporting genetic segregation or functional experiments. We provided for the first time evidence of a variant in *TUBA4A* that segregates with disease in a FTD family, identified by using whole exome sequencing. Neuropathological examinations showed numerous transactive response DNA-binding protein 43 positive dystrophic neurites and relatively lower number of neuronal cytoplasmatic inclusions. Our findings support that *TUBA4A* variants may be a rare genetic cause of familial FTD.

Introduction

Frontotemporal dementia (FTD) is the second most common type of early-onset dementia, typically characterized by behavioral changes and cognitive impairment.[1] Up to 15% of FTD patients develop amyotrophic lateral sclerosis (ALS), while approximately 50% of ALS patients exhibit cognitive symptoms. Due to clinical, genetic and pathological overlapping features, both disorders are considered as part of a disease continuum.[2]

FTD has been found to have a strong familial component with a positive family history in 30-50% of patients.[3] Mutations in microtubule-associated protein tau (*MAPT*), granulin (*GRN*) and Chromosome 9 open reading frame 72 (*C9orf72*) have been shown to be major genetic causes in familial FTD, with *C9orf72* repeat expansion as most common genetic cause for FTD and ALS.[4] Additionally, other gene mutations such as Valosin-containing protein (*VCP*), TAR DNA-binding protein (*TARDBP*) and TANK-binding kinase 1 (*TBK1*), have also been reported to cause FTD and/or ALS.[4] Among these discoveries, variants in Tubulin alpha 4a (*TUBA4A*) were identified in an exome-wide rare variant analysis of ALS cases,[5] and have only sporadically been reported in FTD patients without ALS.[6] However, the association with pure FTD phenotype remains weak due to lack of replication studies and segregation analysis. In this paper, we describe the clinical and pathological features of a genetically unresolved FTD family. Whole-exome sequencing (WES) revealed a novel *TUBA4A* variant, cosegregating with the disease in the family with FTD. Neuropathological examination showed numerous transactive response DNA-binding protein 43 (TDP-43) positive neuronal cytoplasmic inclusions (NCI), dystrophic neurites (DN) and sporadic neuronal intranuclear inclusion (NII). TUBA4A inclusions were not present. Our findings suggest that *TUBA4A* might be involved in the pathogenesis of FTD.

Materials and methods

Subjects

We studied a three-generation Dutch family with nine patients presenting with dementia and/or parkinsonism (Figure 1). FTD without any signs of ALS was diagnosed in three patients (II:8, III:6, III:8), Alzheimer's disease (AD) in one (II:9), Parkinson's disease (PD) in one (II:2) and dementia not otherwise specified in four (II:1, II:3, III:1, III:5). Detailed clinical information of the patients was obtained by interviewing patients and/or their relatives, and by reviewing medical records from hospitals or nursing homes. Medical information was limited in three patients (II:1, II:2, II:3). Available unaffected relative of the proband aged >70 (III:2) was also included. DNA was obtained from blood in participants, and from formalin fixed paraffin-embedded lymph node tissue of one patient (III:1). Pathological confirmation of frontotemporal lobar degeneration (FTLD)

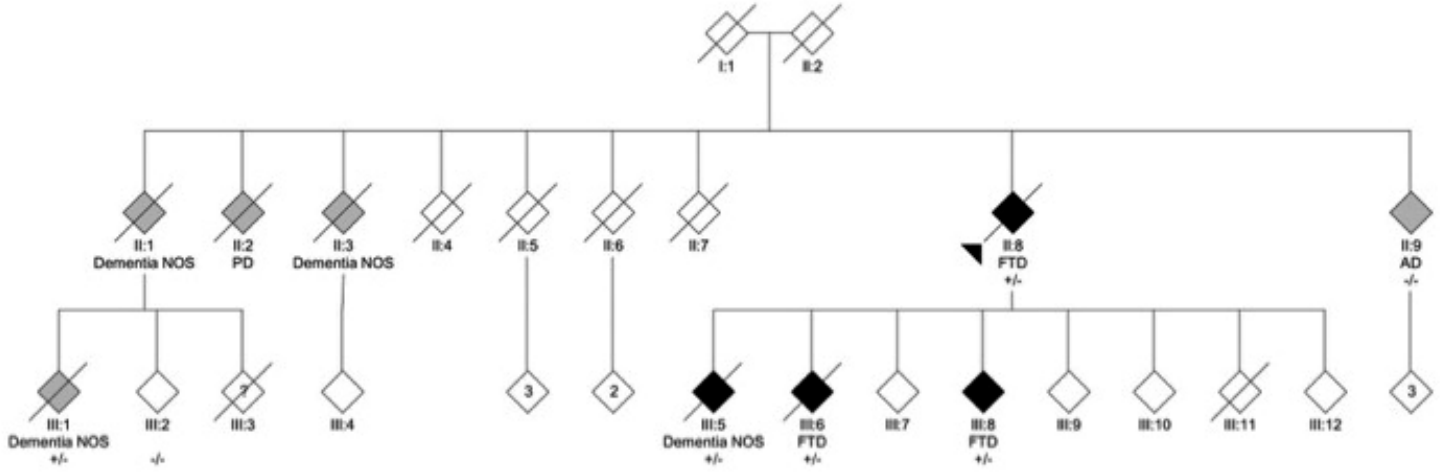

Figure 1. Pedigree of the family with FTD and/of parkinsonism. The proband is indicated with arrowhead and the only individual with pathological examination. Filled black symbols represent affected individuals who were personally examined. Filled grey symbols are affected individuals based on medical records. +/- indicates individuals carrying the *TUBA4A* variant; -/- indicates that the variant is absent; bvFTD=behavioural variant of frontotemporal dementia. AD=Alzheimer's disease. Dementia NOS=dementia not otherwise specified, PD=Parkinson's disease.

was obtained from the proband. The study was approved by the Medical Ethical Committee of the Erasmus Medical Centre, and informed consent was obtained from all participants. For subsequent mutational analysis of *TUBA4A*, we screened a cohort of 59 FTD patients by WES.

Genetic and bioinformatic analysis

Mutation in *MAPT*, *GRN* (by Sanger) sequencing and *C9orf72* repeat expansion (by repeat-primed PCR) were excluded in the proband (II:8) and one offspring (III:6). Five affected individuals (II:8, III:1, III:5, III:6, and III:8) and one unaffected relative (III:2, aged >70) were selected for whole exome sequencing, performed by Centogene AG (Rostock, Germany). Exome capture was carried out with the Nextera Rapid Capture Exome Kit (Illumina, Inc., San Diego, CA). The kit covers 214,405 exons with a total size of about 37 Mb. Sequencing was done using HiSeq4000 sequencers (Illumina) to produce 2 × 150-bp reads, and pooling up to nine exomes per lane. The bioinformatics pipeline was based on the 1000 Genomes Project data analysis pipeline and on Genome Analysis Toolkits (GATK) best practice recommendations.[7,8] In short, raw sequencing data were first converted to standard fastq format using bcl2fastq (Illumina 2.17.1.14), and then aligned to the GRCh37 (hg19) build of the human reference genome using Burrows–Wheeler Aligner software.[9] Alignments were converted to binary bam file format, sorted on the fly and de-duplicated without intermediate input-output-operations to temporary files to achieve maximal performance. To optimize yield, variants were only considered with quality score >200 and coverage depth >50. Variants were annotated using ANNOVAR.[10] Filtering was applied to only include the following variants: 1) segregating heterozygous with the disease; 2) nonsynonymous or protein truncating, including splice-site, nonsense or frameshift indels; 3) minor allele frequency of <0.01% in Genome Aggregation Database (GnomAD);[11] and 4) with a Combined Annotation Dependent Depletion (CADD) score (v1.4) >15.[12] The remaining candidate variants were validated by Sanger sequencing on an ABI3730xl genetic analyzer (Applied Biosystems, CA, USA).

Histology and immunohistochemistry

Neuropathological examination was performed on the proband. Brain autopsy was performed within four hours after death. Tissue blocks were taken from all cortical areas, hippocampus, amygdala, basal ganglia, thalamus and subthalamic nucleus, mesencephalon, pons, medulla oblongata, cerebellum and upper cervical spinal cord, and were embedded in paraffin blocks. Routine staining with haematoxylin and eosin, Congo red, Bodian and methenamine silver was performed. Additional staining was done using the following antibodies: hyperphosphorylated tau (AT-8, Innogenetics; 1:400), phospo (p) transactive response DNA binding protein of 43 kD (TDP-43) (anti p-TDP-43, Cosmo Bio, 1:100 and Proteintech Group, 1:100), p62 (mouse D3 Clone, Santa Cruz, 1:100), TUBA4A (Abgent, 1:100).

The pathological diagnosis of FTLD was made by neuropathologist (A.J.M.R.) and TDP-43 pathology was classified in subtypes described by Mackenzie et al.[13, 14]

Results

Clinical and neuropathological findings

Main clinical features are summarized in Table 1. The mean age at onset of all patients was 66,6 years, and early onset dementia was found in three patients (II:8, III:6 and III:9). The mean age at death was 74,6 years.

Behavioral changes, including disinhibition, emotional blunting and self-neglect, were the initial clinical presentations for most patients (II:8, III:1, III:6, III: 8), except for one (III:5) who presented with memory complaints. Early extrapyramidal symptoms including rigidity, stiff gait and/or postural instability were observed in a few patients (III:5, III:6 and III:8). However, treatment with levodopa did not improve the parkinsonian symptoms of patient III:5. Symptoms consistent with ALS were absent in all patients. Neuropsychological assessment revealed a frontal syndrome with apathy, deficits in language and executive functions, and relatively mild memory deficits in three patients (II:8, III:6, III:8). Brain MRI showed mild generalized atrophy in patients III:5, III:6 and III:8, and moderate hippocampus atrophy in patients III:5 and III:8. SPECT showed hypoperfusion in frontotemporal regions in the proband (II:8) most prominent on the right side. 18F-fluorodeoxyglucose PET showed frontal hypometabolism in two patients (III:6 and III:8), and normal AD CSF biomarkers, ruling out AD.

Genetic analysis

Mutations in the FTD and AD causing genes (*C9orf72*, *GRN*, *MAPT*, *VCP*, *FUS*, *TARDBP*, *TBK1*, *SOD1*, *SQSTM1*, *APP*, *PSEN1*, *PSEN2*) were excluded. After filtering, we found four heterozygous candidate variants in four genes (*TUBA4A*, *ZNF142*, *PTPRE*, and *ARAP3*). All variants were confirmed by Sanger sequencing, and co-segregated with disease in the family. We nominated missense mutation in *TUBA4A*, c.313C>T (p.R105C) located in exon 3, as top candidate gene, based on its genetic association with ALS.[5] This novel variant has not been reported in gnomAD and in house exome data. In silico prediction tools predict a damaging effect in all tools with a CADD score of 33.

At protein level, the variant is located in the GTPase domain. The mutation p.R105C changed polar with a positive side-chain for a non-polar with a neutral side-chain. This could affect the formation of tubulin, and as a subsequent the forming of microtubules.

We did not identify additional variants in the WES data of a cohort of FTD patients (n=59).

Table 1. Summary of demographics and clinical symptoms of the affected individuals

ID	Age at onset	Disease duration	Current age /age at death[#]	Clinical diagnosis	Parkinsonian symptoms	Imaging	TUBA4A carrier
II:1*	70	6	76[#]	Dementia NOS	N/A	N/A	N/A
II:2*	66	6	72[#]	PD	+	N/A	N/A
II:3	68	9	77[#]	Dementia NOS	N/A	N/A	N/A
II:8*,[1]	64	11	75[#]	bvFTD	N/A	SPECT: ↓ FT perfusion	+
II:9	74	16	90	AD	-	MRI: MTA 2 bilateral	-
III:1	68	9	77[#]	Dementia NOS	N/A	N/A	+
III:5*	65	6	71[#]	Dementia NOS	+	MRI: mild generalized atrophy, MTA 2 bilateral	+
III:6*	64	6	70	bvFTD	-	MRI: mild generalized atrophy PET: ↓ frontal uptake	+
III:8*	59	8	67	bvFTD	+	MRI: mild generalized atrophy PET: ↓ frontal uptake	+

Clinical diagnosis: Dementia NOS, dementia not otherwise specified; bvFTD, behavioural frontotemporal dementia; AD, Alzheimer's disease; PD, Parkinson's disease. *Imaging:* FT, frontotemporal; MTA= medial temporal atrophy; N/A, Not available; +, present; -, absent; *individuals selected for exome sequencing; [1] Neuropathological confirmed FTLD-TDP. [#] Age at death.

Neuropathology

Brain autopsy was performed in the proband (brain weight 1216 gr). Neuropathological examination showed moderate bilateral frontal atrophy, with microscopically slightly gliosis of the first layer and moderate spongiosis in the second layer of the frontal cortex, accompanying with severe neuronal loss in entorhinal cortex, and more prominently in

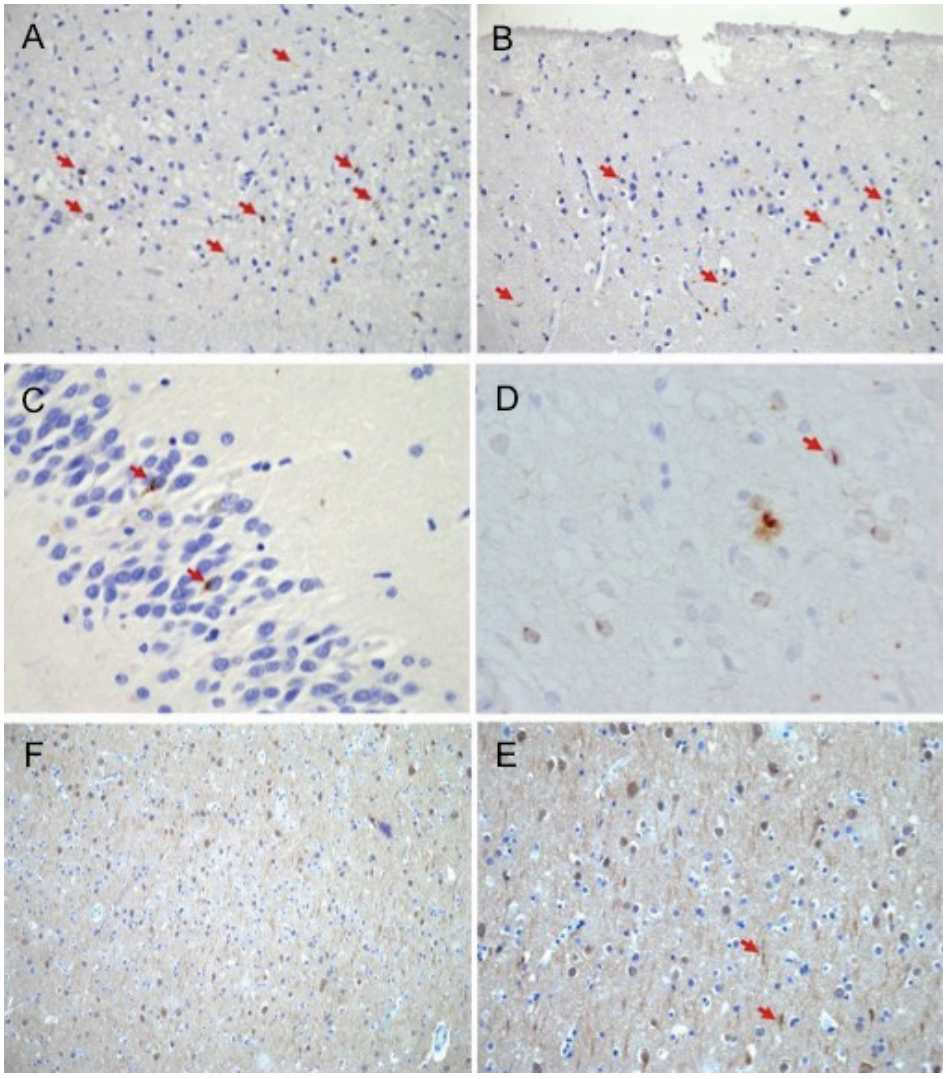

Figure 2. Neuropathological features of the proband (II:8) with *TUBA4A* variant. Numerous pTDP-immunoreactive (IR) dystrophic neurites and relatively lower number of neuronal cytoplasmatic inclusions (NCI) were found in the frontal and temporal cortex **(A and B)**. A few pTDP-IR NCI is observed in dentate gyrus **(C)**. Lentiform neuronal intranuclear inclusions were sporadically found in the temporal cortex **(D)**. TUBA4A staining showed no neuronal inclusions in temporal cortex **(E)**, and only some faint staining of neuronal cytoplasm and its axons and dendrites **(F)**.

CA1 and subiculum. The substantia nigra showed severe loss of pigmented neurons. No Lewy bodies were found, neither in locus coeruleus or pons. Abundant p62 and pTDP immunoreactive (IR) short DN, but relatively lower number of NCI, were observed predominantly in the superficial layers of temporal and frontal cortices (Figure 2A and 2B), and in a lesser extent in the parietal cortex. A few NCI were also found in the dentate gyrus (Figure 2C), caudate and putamen. Lentiform NII were sporadically found in the cortical regions (Figure 2D). Moderate glial cytoplasmatic inclusions were seen in the white matter of frontal, temporal and parietal cortices. Low numbers of AT8 positive neurofibrillary tangles and neuropil threads were found in the hippocampus accompanying with some plaques in the cortical regions, consistent with Braak stage 2 and amyloid B.[15] The neuropathological diagnosis was FTLD-TDP with mixed of type A and B.

Immunohistochemistry with TUBA4A antibodies revealed faint staining of neuronal cytoplasm including its axons and dendrites, but no TUBA4A-IR inclusions (Figure 2E and 2F). Similar findings was observed in other FTLD-TDP (type A and type B) cases and non-demented controls.

Discussion

This is the first study providing evidence of cosegregation of a novel variant (p.R105C) in *TUBA4A* with disease in a family with FTD without any symptoms consistent with ALS. The clinical presentation in this family is heterogeneous, including FTD, Parkinson's disease and dementia with parkinsonism. The mutation is predicted as damaging by in silico tools, and may disrupt a proper forming of microtubules. TDP-ir NCIs and DNs mainly in the frontal and temporal cortices and sporadic NII are the neuropathological findings of one *TUBA4A* variant carrier.

The frequency of *TUBA4A* variant is assumed to be rare in familial FTD as no additional variant in *TUBA4A* has been found in our Dutch cohort. To date, only a few rare variants in *TUBA4A* have been reported in FTD or FTD-ALS patients without supporting cosegregation or neuropathology.[5, 6] Furthermore, one study has failed to identify any disease causing *TUBA4A* variants in a large cohort of familial and sporadic FTD,[16] raising the question whether *TUBA4A* variants are involved in the pathogenesis of FTD. Although we provide segregation with disease in our FTD family, the pathogenicity of this variant is still uncertain due to lack of supportive functional experiments. Furthermore, the variant in the present study is located in exon 3 encoding the GTPase domain, which is in contrast with the reported pathogenic variants in *TUBA4A* in exon 4 that encodes the Tubulin domain.[5, 17] At last, we could not rule out the possibility that one of the other three shared variants, identified by WES, is the true causative mutation in our FTD family. Therefore, functional experiments are still warranted to investigate the potential role of this variant on microtubule function and its relation with FTD.

Behavioral changes consistent with behavioral FTD, including parkinsonian symptoms in two patients, without symptoms suggestive for ALS were main clinical presentation among the *TUBA4A* carriers in our family. Besides, one family member was diagnosed with PD, although no medical history was available. Parkinsonism is a common clinical presentation for FTD patients, in particularly in bvFTD, and has been observed in up to 38.7% of patients.[18, 19] Various motor symptoms have been reported in FTD patients, with bradykinesia as most frequent (up to 84%), followed by parkinsonian gait and postural instability.[18] Motor symptoms are usually unresponsive for levodopa treatment. Clinical overlap exists with other hyperkinetic movement disorders such as PD, progressive supranuclear palsy (PSP) and corticobasal syndrome (CBS). An association has been found between motor symptoms and genetic mutations, with PSP most commonly associated with *MAPT* mutation carriers, and CBS with *GRN* mutation carriers. Interestingly, behavioral FTD with motor symptoms (including positive glabellar sign, discrete stooped posture with decrease armswing) have also been reported in a *TUBA4A* p.Arg64Glyfs*90 carrier with positive family history for PD before the age of 65. These observations support that *TUBA4A* variant may be associated with bvFTD with parkinsonism.

The neuropathological finding of many short pTDP-ir DN but relatively lower number of NCI and glial inclusions complicated the classification into one of the TDP subtypes.[13] The presence of NCI and short DN, mainly in the superficial layer, is characteristic for TDP type A, but the relatively low number of NCI with rare NII is atypical, and would resemble TDP type B. This indicates that *TUBA4A* variants may be associated with FTLD-TDP subtype with characteristics of both TDP type A and B. None of the neuronal inclusions were positively stained with TUBA4A antibody. As no other neuropathology of *TUBA4A* carriers has been reported so far, additional neuropathological examinations of FTD patients with *TUBA4A* variants are needed to investigate this association.

TUBA4A encodes for the protein α-tubulin, which forms a heterodimer with β-tubulin into microtubules. Microtubules are essential for neuronal morphogenesis and function.[20] In vitro studies by transfection of several *TUBA4A* mutants associated with ALS showed a reduced ability to form tubulin dimers with decreased incorporation into the microtubules in primary motor neurons and HEK293 cells for some mutants.[5] In COS7 cells, a disruption of microtubule dynamics and stability in has been observed. These observations suggest a dominant-negative mechanism resulting in decrease microtubule function. The question remains whether the *TUBA4A* variant in the present study will follow the same pathomechanism as observed by Smith et al, because of distinct location of the variant and clinical presentation. Functional studies are needed to investigate the effect of our variant on microtubule network and to elucidate the disease mechanism.

In conclusion, our data strongly supports the association of *TUBA4A* with pure FTD, and provides for the first time, evidence of cosegregation and neuropathology of a *TUBA4A*

carrier. However, functional studies are still needed to investigate the pathogenicity of this variant. Together with previous reports, *TUBA4A* mutation is assumed to be a rare cause for FTD.

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CHAPTER 3.3

3.3

PRKAR1B mutation associated with a new neurodegenerative disorder with unique pathology

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Abstract

Pathological accumulation of intermediate filaments can be observed in neurodegenerative disorders, such as Alzheimer's disease, frontotemporal dementia and Parkinson's disease, and is also characteristic for neuronal intermediate filament inclusion disease. Intermediate filaments type IV include three neurofilament proteins (light, medium and heavy molecular weight neurofilament subunits) and α-internexin. The phosphorylation of intermediate filament proteins contributes to axonal growth, and is regulated by protein kinase A. Here we describe a family with a novel late-onset neurodegenerative disorder presenting with dementia and/or parkinsonism in 12 affected individuals. The disorder is characterized by a unique neuropathological phenotype displaying abundant neuronal inclusions by hematoxylin and eosin staining throughout the brain with immunoreactivity for intermediate filaments. Combining linkage analysis, exome sequencing and proteomics analysis, we identified a heterozygous c. 149 T>G (p.Leu50Arg) missense mutation in the gene encoding the Protein kinase A type I-beta regulatory subunit (*PRKAR1B*). The pathogenicity of the mutation is supported by segregation in the family, absence in variant databases, and the specific accumulation of PRKAR1B in the inclusions in our cases associated with a specific biochemical pattern of PRKAR1B. Screening of *PRKAR1B* in 138 patients with Parkinson's disease and 56 patients with frontotemporal dementia did not identify additional novel pathogenic mutations.

Our findings link a pathogenic *PRKAR1B* mutation to a novel hereditary neurodegenerative disorder and suggest an altered protein kinase A function through a reduced binding of the regulatory subunit to the A-kinase anchoring protein and the catalytic subunit of protein kinase A, which might result in subcellular dislocalization of the catalytic subunit and hyperphosphorylation of intermediate filaments.

Introduction

Neurofilament proteins assembling into neuron-specific intermediate filaments (IF) type IV, are major constituents of the axonal cytoskeleton. Neurofilaments undergo significant changes in their subunit composition during development and in adult neurons, and play an essential role in axonal growth, axonal transport and signalling pathways.[1] In the CNS, the major neuronal IF can be distinguished into three neurofilaments proteins: NF-L (light), NF-M (medium) and NF-H (heavy), and α-internexin, each composed of an N-terminal head domain, an α-helix-rich central rod domain, and a C-terminal tail domain.[2] Phosphorylation of the Lys-Ser-Pro repeats sites at the C-tail of NF-H and NF-M and at sites at the N-terminal domain, was proven to be essential for neurofilament-specific function.[3] The cyclic AMP-dependent protein kinase A (PKA) plays a major role in phosphorylation of neurofilaments,[4] and hyperphosphorylation of Lys-Ser-Pro repeats sites causes disrupted neurofilament axonal transport, prevents turnover of neurofilaments by ubiquitin proteasome system, and results in the accumulation of neurofilaments.[4-7]

PKA is an heterotetramer, consisting of two regulatory and two catalytic subunits, which is inactive in the absence of cyclic AMP.[8] Binding of cyclic AMP to regulatory subunits unleashes the catalytic subunit, thereby enabling PKA signalling. The regulatory subunits also provide binding sites for A-kinase anchoring protein (AKAP), a scaffold protein for targeting PKA signalling.[9, 10] Mutation in these regulatory subunits are shown to alter the PKA function.[11, 12]

Several neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, motor neuron disease, and frontotemporal dementia (FTD) show aggregation of neurofilaments in association with disease-specific accumulation of tau, α-synuclein or transactive response DNA-binding protein 43 (TDP-43), respectively.[5] Neuronal intermediate filament inclusion disease (NIFID), a rare neurodegenerative disorder, shows NF and fused in sarcoma (FUS) protein-positive inclusions, which are negative for tau, TDP-43 and α-synuclein.[13-18] NIFID is a non-familial disorder,[16, 19, 20] and neither pathogenic variants in any of the genes coding for IFs and FUS, nor biochemical modifications of IF were found.[15, 21, 22]

Furthermore, neurofilaments inclusions are found in patients with Charcot-Marie-Tooth disease type 2E and in transgenic mice with a mutation at the major phosphorylation site (Ser55Asp) of *NEFL* gene.[23-25] Also, a transgenic mouse model overexpressing α-internexin, induces the formation of cerebellar torpedoes, and abnormal accumulation of neuronal IF.[26]

In this paper, we report a novel familial neurodegenerative disorder with a highly specific neuropathological phenotype consisting of abundant α-internexin-positive, but

FUS-negative neuronal inclusions. By means of genome-wide linkage analysis, exome sequencing and proteomics of neuronal inclusions, we have identified a pathogenic mutation in the gene coding for the type I-beta regulatory subunit of protein kinase A, *PRKAR1B*. The mutant protein is found to be associated with aggregates of IF in this disease.

Materials and methods

Subjects details

We studied a three generation-large family with twelve affected patients presenting with dementia and/or extrapyramidal syndrome (Fig. 1 and Table 1). Medical information was limited in the five deceased affected members (II:1, III:1, III:12, III:13, III:14). One clinically unaffected patient, III:9, who died from myeloid leukemia at 57 years old, has been described with α-synuclein negative Lewy bodies previously.[27] The proband (III:4) from this family was evaluated for motor and mental complaints at the age of 56 years. The age at onset of the affected individuals varied between 45 and 64 years, the mean disease duration was 14.8 years, but varied from 5 to 25 years.

Table 1. Clinical characteristics of the individuals in the family

Subject	Age at onset	Age at death	Duration	Current age	Dementia	Parkin-sonism	Atrophy on MRI/CT
II:1	45	70	25	-	+	+	NA
III:1	NA	NA	NA	NA	-	+	NA
III:2[a]	50	67	17	-	+	_	NA
III:3	50	75	25	-	+	NA	Frontal
III:4[a]	56	61	5	-	+	+	Generalised
III:5	-	-	-	65	-	-	NA
III:6	60	-	-	67	+	+	Generalised
III:7	<57	63	NA	-	+	NA	NA
III:8	<60	62	NA	-	+	NA	NA
III:9[a]	-	57	-	-	-	-	NA
III:10	63	71	8	-	+	+	Generalised
III:11	-	-	-	80	-	-	NA
III:12	<65	67	NA	-	+	NA	NA
III:13	64	73	9	-	+	NA	Generalised
III:14	NA	74	NA	-	+	NA	NA
Range	45-64	61-75	5-25				

+ or – indicate the presence or absence of the phenotype or information; NA, not available.
[a] Autopsy confirmed cases.

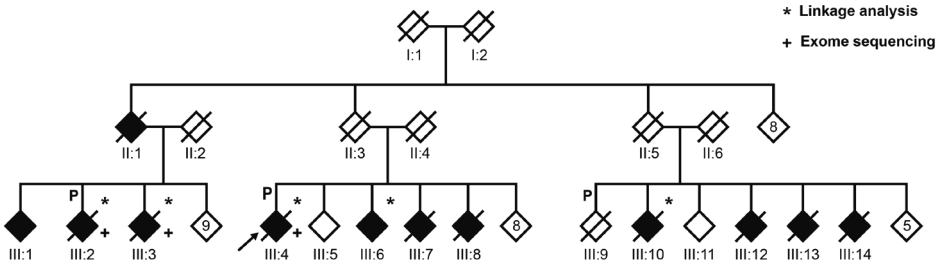

Figure 1. Pedigree of the family with *PRKAR1B* mutation. Filled symbols represent affected individuals by behavioural symptoms, dementia and or parkinsonism, and empty symbols represent unaffected individuals. Symbols with a diagonal line represent deceased individuals. Pathology is denoted by P, and the proband is indicated by an arrow. Numbers in symbols indicates the number of individuals. Sex of the pedigree members are obscured to protect privacy. * = individuals included in linkage analysis; + = individuals included in exome sequencing.

DNA was extracted from peripheral blood samples of patients III:2, III:3, III:4, III:5, III:6, III:10, III:11, and from the spleen of patient III:9. The study was approved by the Medical Ethical Committee of the Erasmus Medical Center Rotterdam, and all family members participating in the study or their legal representatives have given informed consent. A series of 138 unrelated patients with Parkinson's disease and an autosomal dominant pattern of inheritance were also studied. The clinical diagnosis of Parkinson's disease was established according to widely used criteria.[28] The patients originated from Italy (*n*=114), Brazil (*n*=14), Portugal (*n*=9) and The Netherlands (*n*=1). The average onset age of Parkinson's disease symptoms was 53.5 ± 11.7 years (range 20-75), and the average disease duration was 8.5 ± 7.1 years (range 1-36). In these patients the entire *PRKAR1B* coding region (10 exons) and exon-intron boundaries were sequenced by Sanger protocols in order to find possible pathogenic variants. Primers and PCR protocols are reported in the Supplementary Table 1. *PRKAR1B* variants were also analyzed in exome sequenced cohorts of familial FTD (*n*=51) and FTD-FUS (*n*=5). The average onset age of symptoms was 58.4 ± 8.6 years (range 36-73) in familial FTD and 33.7 ± 2.4 (range 30-36) in FTD-FUS. The average disease duration was 66.8 ± 8.8 (range 43-83) in familial FTD and 43.1 ± 3.7 (range 39-46) in FTD-FUS.

Genomic analysis

CSV files containing SNP call data from HumanCytoSNP-12v2.1(Illumina) arrays of five related patients, see Fig. 1, were adapted by GenomeStudio (Illumina) for linkage analyses using Allegro[29] implemented in easyLINKAGE Plus.[30] SNPs with a call rate lower than 95% were excluded from the calculations. Mendelian inheritance check was performed for all family members, with the program PedCheck.[31] SNPs showing Mendelian inconsistencies were excluded from the calculation. Individuals who were encoded by the pedigree information file were used for allele frequencies computation.

Two separate multipoint linkage analyses were performed (affected only) on genotypes from five affected individuals using Allegro with a SNP spacing of 0.2 cM and one of 0.5 cM. LOD scores in sets of 100 markers were calculated assuming the disease to be an autosomal dominant disorder with a gene frequency of 0.0001 in the population. Regions showing a LOD score > 1.5 in both models were used as candidate regions (Supplementary Fig. 1). As borders, flanking SNP markers were used. Additionally, genome wide copy number analysis in genotyped individuals was performed using signal intensity files generated with GenomeStudio 2011, V2011.1 (Illumina, San Diego, CA) in Nexus Copy Number, Discovery Edition, ver. 5.1 (BioDiscovery, El Segundo, CA).

Three individuals (III:2, III:3 and III:4) were selected for exome sequencing (Fig. 1). Whole exome capture and sequencing were performed by LGC Genomics GmbH (Berlin, Germany). Exomes were captured by Agilent's SureSelect AllExon Kit, and were sequenced with 100 base pair reads on the Illumina HiSeq2000 platform, according to the manufacturer's protocol. Reads were mapped to the human reference genome sequence (assembly GRCh37/hg19) using the Burrows-Wheeler Alignment Tool.[32] The identified variants per individual were called by using Genome analysis Tool Kit (GATK) and annotated by ANNOVAR.[33, 34] GATK was also used for base quality recalibration, local sequence realignment and variant filtering to minimize base calling and mapping errors. Variants with quality score < 30, quality over depth < 5, strand bias > -0.10 and depth < 20 were filtered out. Additionally, Indels with strandbias > -1.0 instead of -0.10 were filtered out. We used the dbSNP129 (http://www.ncbi.nlm.nih.gov/projects/SNP/), the 1000 genome project (www.1000genomes.org/) and the National Heart Lung Blood Institute Exome Variant Server (https://evs.gs.washington.edu/EVS/) to filter out polymorphisms. The predicted functional effects of the novel sequence variants were assessed by Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/), Sorting Intolerant from Tolerant (SIFT) (http://sift.jcvi.org/www/SIFT_enst_submit.html), PROVEAN (http://provean.jcvi.org/seq_submit.php) and Mutation Taster (www.mutationtaster.org). The conservation of amino acid across different species was identified by MUCLES.[35]

Histology and immunohistochemistry

The Netherlands Brain Bank performed brain autopsy (III:2 and III:4) within four hours of death according to their Legal and Ethical Code of Conduct of the Netherlands Brain Bank. Tissue blocks were taken from all cortical areas, hippocampus, amygdala, basal ganglia, substantia nigra, pons, medulla oblongata, cerebellum, and cervical spinal cord, and were embedded in paraffin blocks and subjected to routine staining with haematoxylin and eosin, periodic acid-Schiff reaction and silver staining. Brain autopsy and routine staining of the third case (III:9) was described by van Duinen (van Duinen et al., 1999), and several regions were obtained for immunohistochemistry.

Immunohistochemistry was performed with antibodies directed against: hyperphosphorylated tau (AT-8, Innogenetics, Ghent, Belgium; 1:400); β-amyloid protein (DAKO, Glostrup, Denmark; 1:100, following formic acid pre-treatment); α-synuclein (Zymed Laboratories, San Francisco, California, USA; undiluted, following formic acid pre-treatment); poly-ubiquitin-binding protein p62 (BD Biosciences Pharmingen, San Diego, CA, USA; 1:200, following pressure cooking); TDP-43 (ProteinTech Group, Chicago, IL, USA; 1:100, following pressure cooking); neuroserpin (Abcam; 1:100); SMI-31 (Sternberger, Lutherville, MA; 1:5000); SMI-32 (Sternberger; 1:7000) α–internexin (Invitrogen, Camarillo, CA, USA, 1:100, following pressure-cooking); FUS (ProteinTech; 1:25–1:200 with initial overnight incubation at room temperature, following pressure cooking), and PRKAR1B (2x anti-PRKAR1B, Abcam ab38225; 1:50 and Santa Cruz Biotechnology SC-907, Inc; 1:125). Specificity of PRKAR1B antibody is described in other studies.[36, 37] Primary antibodies were incubated overnight at 4°C followed with BrightVision horseradish peroxidase-linked secondary antibody (Immunologic). The immunoreactivity was visualized by freshly prepared Liquid DAB Substrate Chromogen solution (DAKO). Slides were counterstained with Mayer's haematoxylin and mounted in Entellan.

Double immunofluorescence staining

For double staining, autofluorescence of brain tissue was quenched by treatment with 0.1% Sudan Black B (Sigma-Aldrich) in 70% ethanol. Secondary antibodies were Cy3-conjugated anti-mouse (Jackson ImmunoResearch; 1:100) and Alexa 488-conjugated anti-rabbit secondary antibody (Invitrogen; 1:100). Slides were mounted in Mowiol and analysed by confocal microscope (Leica).

Electron-microsocopy

Minute pieces of frontal cortex were fixed in 4% glutaraldehyde in 0.1 M phosphate (pH 7.2), postfixed in 1% osmiumtetroxide and embedded in Epon. Semithin sections (1 µ) were stained with toluidine blue. Areas of interest were selected for ultrathin sectioning (50-60 nm). Contrast was enhanced by staining with lead citrate.

Quantitative proteomics

Approximately 1000 positive inclusions were excised by laser capture microdissection (Carl Zeiss Microscopy, Göttingen, Germany) from each of the two brains (III:2 and III:4). In addition, tissues from the same brain areas of two healthy control brains without inclusions were collected in the same manner. Tissues were lysed in 25 µL SDS sample buffer, separated by 10% SDS PAGE, and stained with colloidal Coomassie Blue. Each gel lane was cut into two gel pieces of equal size, destained, and incubated with trypsin (1 µg, Promega) for 16 hrs at 37°C.

Peptides were separated on a 200 mm reversed phase nano-column (100 µm ID packed with 3 µm Alltima C18 particle from Alltech) using an Eksigent NanoLC ultra system (AB-Sciex). The acetonitrile concentration in 0.1% acetic acid was increased linearly from 4.5% to 38% in 40 min, and to 80% in 1 min. The flow rate was 400 nL/min. The eluted peptides were electro-sprayed into the LTQ-Orbitrap discovery (Thermo Electron). The mass spectrometer was operated in a data-dependent mode, in which one MS full scan (m/z range from 330 to 2000) was followed by MS/MS scans on five most abundant ions. The exclusion window was 25 sec. The mass spectrometric data was searched against the IPI human database (ipi human v3.87) with MaxQuant software (version 1.3.0.5). The search parameters were MS tolerance, 20 ppm; MSMS tolerance, 0.5 Da; enzyme, trypsin with maximum missed cleavages of 2.

Sequential biochemical fractionation and immunoblot analysis

Post-mortem frozen brain tissue from two cases (III:2 and III:4), two frontotemporal lobar degeneration (FTLD) TDP cases and two Alzheimer's disease cases, were dissected, weighted, and sequentially extracted with buffers of increasing strength as previously described.[38] Briefly, grey matter was extracted at 5 mL/g (volume/weight) with 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 (LS + 1% Triton X-100 + 0.5M NaCl), myelin floatation buffer (30% sucrose in LS + 0.5M NaCl), and sarkosyl (SARK) buffer (1% N-Lauroyl-sarcosine in LS + 0.5 M NaCl). The SARK insoluble material was extracted in 0.25 mL/g urea buffer (7M urea, 2M 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 PVDF membranes (Millipore).

Following transfer, membranes were blocked with Tris buffered saline containing 3% powdered milk and probed with the mouse monoclonal antibody PKA [RI] raised against the C-terminal portion (aa 225-381) of the type I regulatory subunit (610166, Becton Dickinson Laboratories), the polyclonal antibody PKA Iβ reg (sc-907 (c-19), Santa Cruz) and a mouse monoclonal anti-α-internexin (32-3600, Invitrogen). Primary antibodies were detected with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch Europe, UK), and signals were visualized by a chemiluminescent reaction (Millipore) and the Chemiluminescence Imager Stella 3200 (Raytest, Switzerland).

For dephosphorylation experiments, urea fractions were dialyzed against RIPA buffer and treated with 400 units lambda phosphatase (New England Biolabs, Ipswich, UK) for 30 min at 30°C.

Results

Clinical and pathological features

Behavioural changes (self-neglect, delusions), with apathy and anxiousness, and memory problems with disorientation, followed by stiffness, shuffling gait, and frequent falls without tremor were the presenting symptoms in most of the patients (Table 1). Impaired attention and concentration, and deficits in memory, language, executive and visuo-constructional functions were found in two neuropsychologically evaluated patients (III:4 and III:6). MMSE and FAB of the proband were respectively 20 and 6, and 18 and 8 for patient III:6. These two patients showed mild to moderate rigidity, bradykinesia, postural instability, small-stepped gait and normal ocular movements, and no cerebellar and motor neuron disease signs at neurological examination on the first evaluation. Unified Parkinson's Disease Rating Scale of the proband showed 6 points in part I, 13 points in part II, 16 points in part III, Hoens and Yahr grade four and Schwab and England ADL scale of 40%. EMG in the proband revealed no evidence for motor neuron disease, myopathy or polyneuropathy. Generalized cerebral atrophy was seen on MRI or CT of the brain in four patients (III:4, III:6, III:10, III:13), and more prominent frontal atrophy in proband (III:4) (Supplementary Fig. 2). Dopaminergic medication in two patients had only a modest effect. Moreover, [18F] fluorodeoxyglucose PET showed frontal hypometabolism in the proband (Supplementary Fig. 2), but a normal FP-CIT scan and normal CSF profile which is incompatible with Parkinson's disease and Alzheimer's disease. Brain autopsy was performed in three patients (III:2, III:4 and III:9).

Macroscopic examination showed mild cerebral atrophy (III:2, III:4, III:9), slightly more pronounced in the frontoparietal cortex in two. An irregular tumor, defined as glioblastoma multiforme in the right temporal lobe was found in the brain of III:4 (absent on MRI one year earlier).

The neocortex showed normal cytoarchitecture in all three brains, with moderate neuron loss in the substantia nigra in two, and severe loss of Purkinje cells in one case. Abundant eosinophilic, periodic acid-Schiff reaction negative, neuronal cytoplasmatic inclusions (NCI) with a glossy weakly stained core were observed in all neocortical regions in layers 3 - 6, hippocampus, substantia nigra, brainstem and spinal cord. Low to moderate number of inclusions were seen in the caudate nucleus, putamen, pallidum and the cerebellum (III:2), and in the dentate gyrus of patient III:9. These inclusions stained strongly with p62 and α-internexin antibodies (Fig. 2A-2C), and less intense with neurofilament antibodies, such as, SMI 31 and SMI 32. Larger inclusions showed a target-like picture with a halo and a weakly stained core, whereas smaller inclusions had a more homogeneous intense staining. Immunohistochemistry was negative for FUS, α-synuclein, AT-8, β-amyloid, neuroserpin, and TDP-43. No intranuclear inclusions were seen.

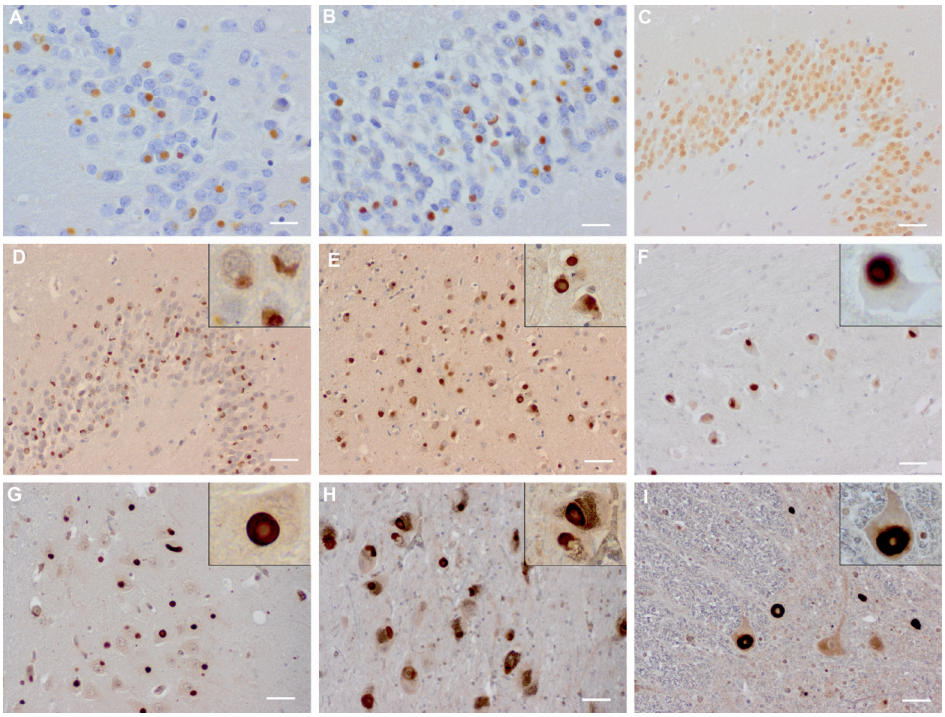

Figure 2. Distribution of neuronal cytoplasmatic inclusions found in familial neurofilamentopathy due to the mutation in the *PRKAR1B* gene. Strong immunoreactivity of neuronal cytoplasmatic inclusions (NCI) with antibodies against α-internexin **(A)** and p62 **(B)** in the dentate gyrus of the hippocampus (HC) is seen in patient III:2. Granular cells of the dentate gyrus show diffuse weak nuclear staining without cytoplasmatic inclusions with FUS antibody **(C)**. PRKAR1B-positive NCI with various sizes are abundant in the HC **(D)** and frontal region **(E)**. A central unstained core surrounded by a strongly immunoreactive halo is found for larger inclusions in different cortices. Many inclusions are also found in the granular layer of the cerebellum **(F)**. The same finding of PRKAR1B-positive NCI is seen in the HC **(G)** of the second patient (III:4). Substantia nigra show moderate neuron loss and positive immunoreactivity for PRKAR1B **(H)**. These inclusions are also seen in lower motor neurons of the spinal cord **(I)**. *Scale Bar:* A and B = 20 µ; C-I = 50 µm.

Genomic and proteomic analysis

Two multipoint linkage analyses of 5 affected patients revealed six regions with LOD score > 1.5 (Supplementary Table 2). No overlapping copy number variant was detected in affected genotyped individuals. Exome sequencing produced approximately 6.4 Gigabases of reads per sample. The average coverage of targeted region was 57x, with 74%, 72%, and 74% covered at least 20x, respectively (Supplementary Table 3). This resulted into a calling of about 48.000 allelic variants per individual after quality filtering using GATK (Supplementary Fig. 3). We examined the exome data on known FTD and PD genes, but no pathogenic variants in known FTD genes (*PGRN*, *MAPT*, *c9orf72*, *CHMP2B*, *VCP* and *FUS*) and PD genes (*LRRK2*, *PARK2*, *PARK7*, *PINK1*, *SNCA*, VPS35, *ATP13A2*, *FBXO7*, *PANK2* and *PLA2G6*) were found.

We performed the genetic analysis in two steps. First, we filtered the exome sequencing data combining with linkage analysis to reduce the number of variants. An analysis of non-synonymous, splice-sites, stop and frameshift variants (SNPs and indels) in the six regions with LOD score > 1.5 showed seven variants shared by the three patients and not annotated by dbSNP129 and having an allele frequency < 1% in 1000 Genomes Project and Exome Variant Server.

Second, we combined proteomics data with the candidate variants found by genetic analysis to identify the causative gene. A total of 1000 inclusions were excised by means of microlaser dissection from fresh-frozen brain samples of patients III:2 and III:4, and corresponding inclusion-free tissue of the same size was obtained from two control brains. The dissection was repeated once from one of the patient samples to obtain a total of 3 replicates. Mass spectrometry of the inclusion bodies was sufficiently sensitive to identify proteins present, but not sufficient to detect protein modifications. Quantitative proteomics analysis revealed six proteins (Polyubiquitin-C, α-internexin, neurofilament light polypeptide, cAMP-dependent protein kinase regulatory subunit type I-beta, neurofilament medium polypeptide and Heat shock cognate 71 kDa protein) that were present consistently and solely in the inclusion samples (Supplementary Table 4).

Combining proteomics analysis with the candidate variants from exome sequencing resulted in one single variant, the novel missense heterozygous variant in *PRKAR1B* on chromosome 7p22 (exon 2: c. 149T>G; p.Leu50Arg; RefSeq NM_002735.2), which is conserved and predicted as pathogenic by four different *in silico* methods that predict functional effects of sequence variations (Fig. 3 and Supplementary Table 5). No novel

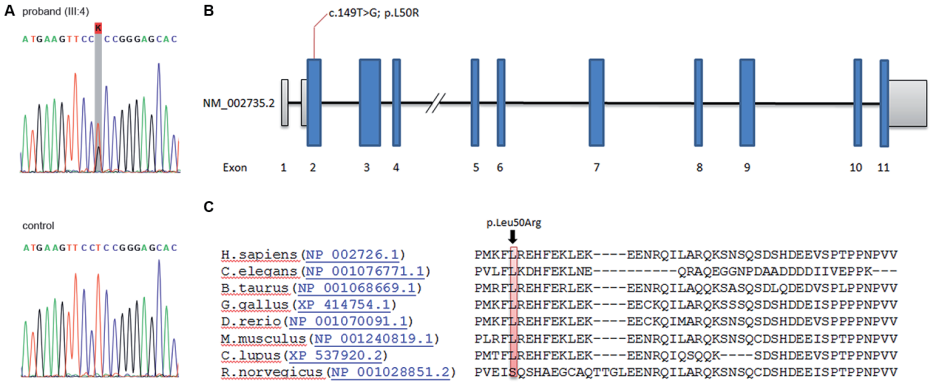

Figure 3. Schematic representation of the *PRKAR1B* genomic structure and conservation of the mutation. Electropherogram showing the *PRKAR1B* mutation at position chr7:750,997 (A>C) in exon 2 (c.T149G; p.Leu50Arg) present in the affected individual (III-4) and absent in an unaffected control of the family **(A)**. Schematic structure of *PRKAR1B* (isoform NM_002735.2) and the position of the mutation identified in the present study is shown. Exons are represented with blue boxes, untranslated regions in gray boxes **(B)**. Alignment of the protein region containing the highly conserved leucine amino acid residue across different species is shown **(C)**. The leucine on position 50 (NP_002726.1) is indicated in the red box.

and pathogenic variants were found in the genes coding for the remaining five proteins identified in the proteomics analysis of the inclusion bodies. Sanger sequencing confirmed the presence of the p.Leu50Arg variant in the affected individuals III:2, III:3, III:4, III:6, III:9 and III:10, and its absence in unaffected family members (III:5 and III:11). Sequencing of the *PRKAR1B* coding region (10 exons) and exon-intron boundaries in a cohort of autosomal dominant Parkinson's disease (*n*=138) and basophilic inclusion body disease (*n*=2) revealed several silent and intronic polymorphisms, which are all annotated in dbSNP, and two non-synonymous rare heterozygous variants, also present in dbSNP (Supplementary Table 6). The first, p.Ile40Phe, was detected in six Parkinson's disease probands (MAF 0.013, similar to dbSNP MAF 0.022), and is considered benign by most of the prediction programs (Supplementary Table 5). The other, p.Arg232Gln, was found in only one Parkinson's disease proband, and is considered pathogenic by all the prediction programs. However, this variant does not co-segregate with Parkinson's disease in the family (one affected sib was not a carrier). This variant is also annotated in dbSNP because of just one allele detected in the 1000 Genome project, and is not present in the Exome Variant Server. Analysis of whole exome sequencing data in a cohort of familial FTD with unknown gene defect (*n*=51) and FTD-FUS (*n*=5) did not reveal any potential pathogenic rare variants in *PRKAR1B* (Supplementary Table 6).

Immunohistochemistry and biochemical analysis

Immunohistochemistry with PRKAR1B antibodies revealed intense staining of the inclusions in brain of the three cases (Fig. 2). Cytoplasmatic inclusions were exclusively seen in neurons and had a variable size. Most inclusions were round and compact with strong PRKAR1B immunoreactivity, and some neurons showed a more granular cytoplasmatic staining (Fig. 2D-I). Round inclusions were seen in the dentate granule cells (Fig. 2D), Cornu Ammonis 1–4, subiculum and entorhinal cortex, and in all layers of the neocortex (Fig. 2E) of the two patients (III:2 and III:4). Many inclusions were also observed in the hippocampus (Fig. 2G), substantia nigra (Fig. 2H), brainstem and spinal cord (Fig. 2I), whereas lower number of inclusions are found in the caudate nucleus, putamen and pallidum. The dentate nucleus and granular layer of the cerebellum showed many cytoplasmatic inclusions in patient III:2 (Fig. 2F), but only some in the same region of patient III:4. Available slices of three brain regions (pons and two neocortex) of patient III:9 also showed abundant PRKAR1B positive inclusions. Overall, more inclusions are found in patient III:2 than in similar regions of the other two cases. Large inclusions showed a faint core with strongly stained halo. The PRKAR1B antibody labelled the inclusions more intensely than p62 and α-internexin. No glial cytoplasmatic inclusions were seen in the cerebral white matter.

Double-labelling immunofluorescence with α-internexin and PRKAR1B antibodies showed that nearly all α-internexin positive inclusions were also PRKAR1B-positive

(Fig. 4A-C). Less than five percent of the inclusions, predominantly those with a smaller size, labelled either for α-internexin or PRKAR1B. Double labelling also showed the spatial relationship of these two proteins within the inclusions; PRKAR1B antibody often labelled the core, whereas α-internexin labelled a halo at the outer side of the inclusions (Fig. 4D-F).

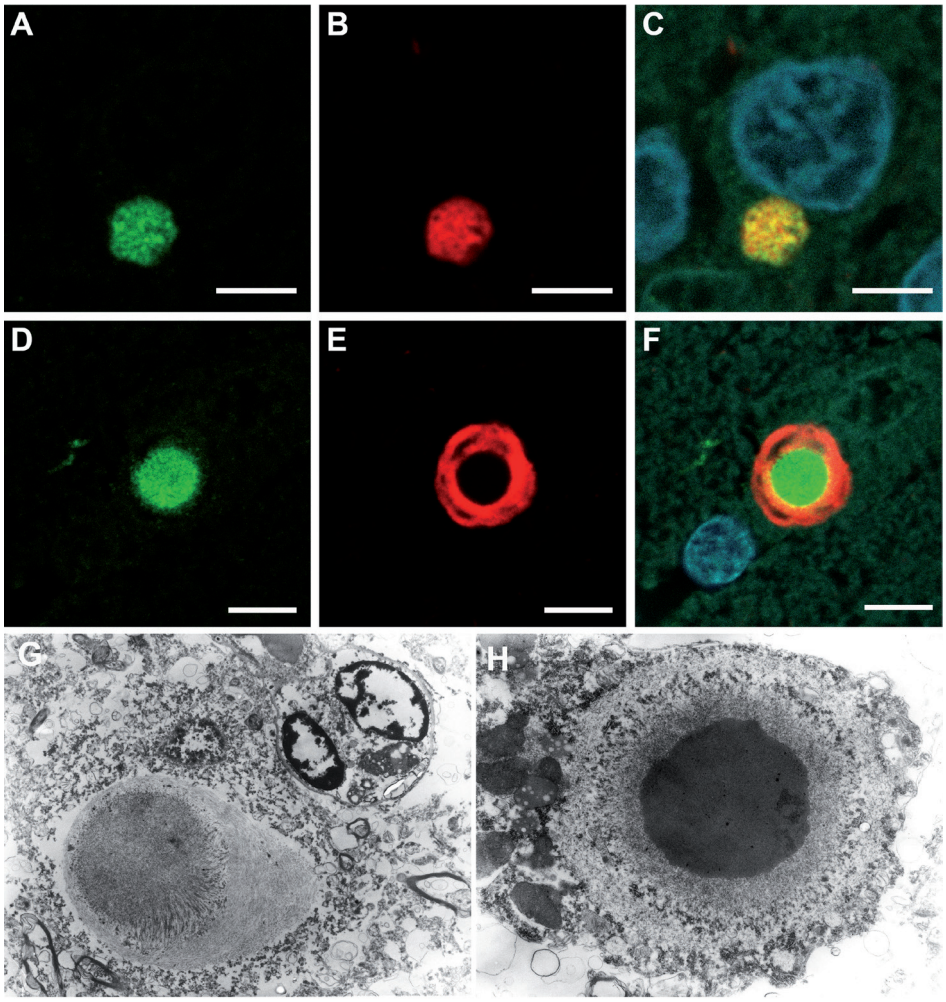

Figure 4. Double-label immunofluorescence for PRKAR1B (green) and α-internexin (red) in familial neurofilamentopathy due to PRKAR1B mutation. Immunofluorescence of the inclusions with PRKAR1B **(A)** and α-internexin [INA] **(B)**. Some inclusions label homogeneous staining with co-localization of INA and PRKAR1B **(C)**. Merged images clearly show that both markers label distinct components of inclusions with a central core labelling for PRKAR1B antibody **(D)** surrounded by a halo labelling for INA **(E, F)**. Scale bar: A and B = 5 μm; D-F = 10 μm. **(G and H)** Ultrastructural examination of inclusions demonstrates dense aggregates of filaments of 11-16 nm surrounded by granule coated fibrils and cellular organelles, G=9000x and H=22500x enlarged.

Ultrastructurally, the core within the body consisted of electron-dense material, and fibrils were found in areas with remnants of vesicles and mitochondria (Fig. 4G and H). The body is composed of dense aggregates of filaments (densely packed fibrils with often a radiating aspect at the edges), while the paler halo consisted of bundles of both circularly and longitudinally arranged filaments.

Neurofibrillary tangles or plaques in Alzheimer's disease (*n*=4), Lewy bodies in Parkinson's disease (*n*=10) and Lewy body dementia (*n*=5), glial inclusions in multiple system atrophy (*n*=4), TDP-43-positive inclusions in FTLD (*n*=4), Pick bodies, Neurofibrillary tangles or pretangles in FTLD with MAPT gene mutations as well as healthy controls (*n*=4), did not show any staining with the PRKAR1B antibody, demonstrating the specificity of PRKAR1B accumulation in our cases (Data not shown).

To characterize potential biochemical alterations of PRKAR1B, proteins were sequentially extracted from temporal cortex from two cases (III:2 and III:4) as well as neurological controls with a series of buffers with an increasing ability to solubilise proteins and analyzed by immunoblot using an antibody that recognizes both isoforms of the type I regulatory subunits of PKA (BD Laboratories).

Two bands at the expected molecular weights of PRKAR1A and PRKAR1B (~48 and 50kDa) were consistently present in the sarkosyl fractions in *PRKAR1B* mutation cases and FTLD and Alzheimer's disease. However, a strikingly different biochemical profile was seen in the urea fractions (fraction enriched for highly insoluble proteins) for the two *PRKAR1B* mutation cases compared to controls (Fig. 5A). Whereas controls showed only minimal reactivity in the urea fraction, a massive enrichment for PRKAR1B was observed in the two cases accompanied by the presence of additional bands of lower and higher molecular weight as well as a high relative molecular mass (M_r) smear. Similar results were observed with a second antibody raised against PRKAR1B (Santa Cruz, data not shown). Since the appearance of higher migrating bands is suggestive for abnormal posttranslational modifications such as hyperphosphorylation, we investigated the phosphorylation state of PRKAR1B by treating dialyzed urea fractions with lambda protein phosphatase. However, this did not reveal any obvious changes in the banding pattern (Supplementary Fig. 4). No biochemical alterations with respect to changes in solubility or appearance of additional bands were observed for α-internexin between our *PRKAR1B* mutation cases and controls (Fig. 5B).

In silico prediction of the PRKAR1B mutation on PRKAR1B protein structure

PRKAR1B codes for the R1β-subunit of cyclic AMP-dependent protein kinase A, which is a tetramer in its inactive form composed of two catalytic and two regulatory subunits (Fig. 6A and B).[39] Binding of cyclic AMP to the regulatory subunits unleashes the catalytic subunits, thereby allowing phosphorylation of PKA substrates. The regulatory subunits

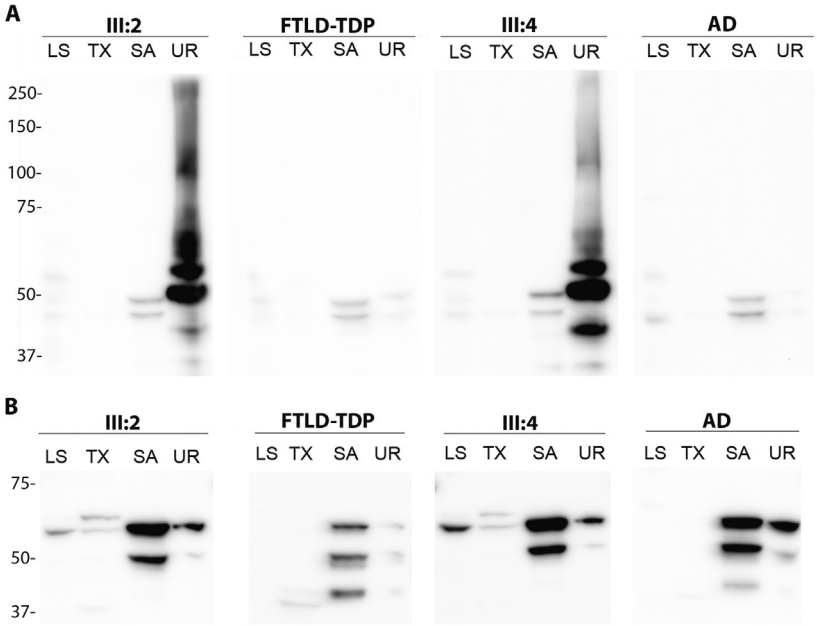

Figure 5. Biochemical analysis of PRKAR1B and α-internexin. **(A)** Proteins were sequentially extracted from temporal grey matter from two cases (III:2 and III:4) with the *PRKAR1B* mutation and neurological controls (Alzheimer's disease [AD] and frontotemporal lobar degeneration with TDP-43 inclusions [FTLD-TDP]). Low salt- (LS), Triton X-100- (TX), sarkosyl- (SA) and urea- (UR) fraction were separated by SDS-PAGE and immunoblotted with anti-PKA RI antibody (BD Laboratories). Cases and controls showed bands in the sarkosyl fraction corresponding to PRKAR1A (~48 kDa) and PRKAR1B (~50kDa). Note the dramatic increase of highly-insoluble PRKAR1B in the UR fraction in cases compared to controls accompanied by appearance of additional bands of ~45kDa and ~55 kDa and a high molecular mass smear. **(B)** The same protein fractions were analyzed by α–internexin immunoblot revealing no obvious changes with respect to changes in solubility or appearance of additional bands between cases and controls.

dimerize via their N-terminal dimerization/docking (D/D) domains. In the inactive PKA tetramer, the D/D domains of the R1β isoform form an integral part of the holoenzyme; while the cyclic AMP binding domains and the linker region tightly interact with one catalytic subunit additional *trans* interactions with the catalytic subunit in the other heterodimer (Fig. 6B). The leucine at position 50 of the PRKAR1B protein is located on the dimer interface formed by the N-terminal D/D domains of the regulatory subunits (Fig. 6A). The leucine side chain forms a hydrophobic core together with several other conserved hydrophobic residues to create this dimer interface (Fig. 6B). A change of leucine to arginine at this position creates a steric hindrance, because due to its larger size the arginine side chain will not fit within the hydrophobic core. Furthermore, the positive charge on the arginine side chain will potentially introduce unfavourable electrostatic interactions with its symmetry-related arginine across the dimer interface. In one scenario, the dimerization interface may still be formed but with local structural

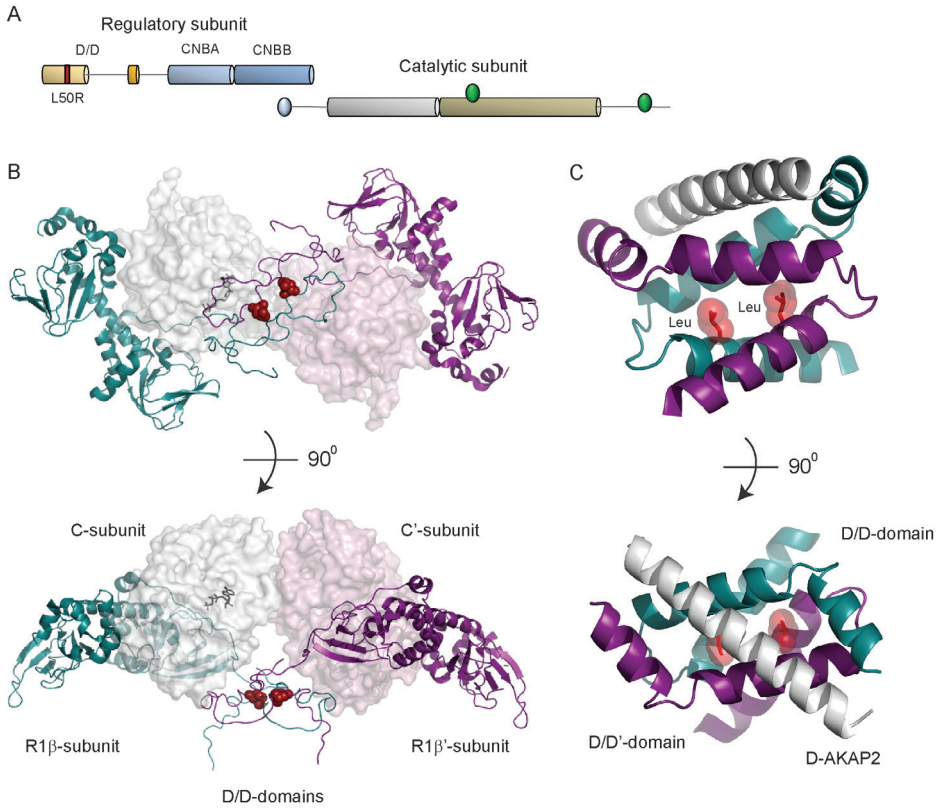

Figure 6. The L50R mutation is located on the interface between the docking/dimerization domains of the protein kinase A regulatory subunit β. **(A)** Organization of the PKA protein subunits. The regulatory subunits contain an N-terminal dimerization/docking (D/D) domain, followed by a linker region and two cAMP binding domains (CNBA and CNBB). The catalytic subunit contains an N-terminal myristylation site for membrane anchoring (blue circle), N-and C-terminal lobes and tails with phosphorylation sites (green circles). The p.Leu50Arg mutation maps to the D/D domain of the regulatory subunit. **(B)** Overall structure of the PKA tetrameric holoenzyme containing two catalytic and two regulatory subunits. The catalytic subunits C (white) and C' (pink) are shown in space filling representation, with ATP bound to subunit C represented in black sticks. The secondary structure elements of the regulatory subunits are shown in cyan (subunit R1β=PRKAR1B) and in purple (symmetry-related subunit R1β') with the leucine on the interface between the D/D domains in red space filling representation. The holoenzyme is shown in two different orientations. **(C)** The importance of the D/D-dimer interface formation for binding to A-kinase anchoring proteins is shown by the structure of the D/D domain of the homologous PRKAR1α subunit in complex with a peptide from Dual-specific AKAP2. Secondary structures are shown in cyan and purple (regulatory subunit D/D domains) and white (Dual-specific AKAP2). Side chains of Leu 50 at the D/D interface are indicated in red stick representation surrounded by transparent spheres. Figure is created using Pymol (deLano Scientific).

rearrangements resulting in an altered conformation. Due to the intimate connections between the D/D domains and the catalytic subunits, these local rearrangements may be propagated throughout the holoenzyme and affect the cyclic AMP-induced response and thus activation of the kinase.[39] Furthermore, the D/D dimerization interface creates

the docking site for AKAPs (Fig. 6C),[40] and local rearrangements may affect AKAP binding and thereby PKA signalling and targeting PKA to a specific subcellular location.[10, 40] In another scenario the arginine completely prevents formation of the dimerization interface between the D/D domains. This would affect correct holoenzyme formation and thereby PKA activation. In addition, the unassembled D/D domain interfaces would expose large hydrophobic areas which are prone to aggregation resulting in insoluble protein.

Discussion

This study describes a novel hereditary neurodegenerative disorder associated with a mutation (c.149T>G; p.Leu50Arg; RefSeq NM_002735.2, NP_002726.1) in the gene coding for the Type I-beta regulatory subunit of the PKA with an unique neuropathological phenotype with PRKAR1B accumulation into abundant neuronal inclusions. The mutation is predicted to prevent or alter dimerization between the D/D domains within the PKA holoenzyme, thereby exposing hydrophobic protein regions that may result in aggregation, or reducing the binding of the regulatory subunits to both the catalytic subunits of PKA, and to AKAP. The frequency of this mutation appears to be rare, as the mutation is absence in dbSNP and Exome Variant Server. Moreover, no pathogenic mutation in *PRKAR1B* could be identified in a cohort of familial Parkinson's disease or frontotemporal dementia.

The present disorder has a rather unspecific phenotype consisting of dementia and parkinsonism with poor response to levodopa, normal FP-CIT scan, and normal CSF biomarkers, which have ruled out Parkinson's disease and Alzheimer's disease. The neuropsychological profile with impairment of multiple cognitive domains and clinical symptoms are consistent with the involvement of all cortical and subcortical regions of the brain. Cerebellar and motor neuron signs were lacking at neurological examination despite the widespread inclusions in cerebellum and spinal cord; however, we cannot exclude that the patients may develop such signs in the last stage of the disease during their stay in a nursing home. Its hereditary occurrence has initially not been recognized,[27] but distinguishes it from the mostly sporadic NIFID. Furthermore, the absence of immunoreactivity with α-synuclein and FUS antibodies distinguishes this disorder from Parkinson's disease and NIFID. The co-occurrence with cancers (myeloid leukemia and glioblastoma) in two out five patients is quite remarkable. PKA stimulates the expression of the NR4 receptor, and NR4 is involved in several malignancies, such as glioblastoma and myeloid leukemia.[41] It might be worth screening for potential variants in *PRKAR1B* in cohorts of patients with these malignancies and to see whether the mutation alters the expression of NR4 receptor. However, this is beyond the scope of this research.

The present approach of combining genome-wide linkage analysis, exome sequencing and proteomic analysis of neuronal inclusions allowed us to identify a heterozygous p.Leu50Arg variant in *PRKAR1B* in five affected family members, consistent with an autosomal dominant mode of inheritance. The highly specific pattern of PRKAR1B accumulation in inclusions in the three autopsy-proven cases together with the dramatic enrichment of PRKAR1B in highly insoluble protein fractions with appearance of abnormal Mr species, the negative PRKAR1B immunoreactivity in any other neurodegenerative disorders (Alzheimer's disease, Parkinson's disease, Lewy body dementia, multiple system atrophy, Pick's disease, and FTLD-TDP-43) and the absence of the mutation in variant databases, strongly argues for a causative role of this mutation in this family.

The pathophysiological mechanism how this mutation leads to neurodegenerative disease remains to be investigated by establishing cell culture and animal models. Our first hypothesis is that the mutation leads to impaired dimerization between the regulatory R1β subunits and catalytic subunits within the PKA holoenzyme. Structural changes within the holoenzyme may liberate the catalytic subunits which could be vulnerable to degradation, resulting in reduced PKA activity. This hypothesis is supported by loss of PKA catalytic subunit and PKA activity shown in PKA regulatory knockout mice.[11] However, catalytic subunits unleashed by mutation-induced structural changes may also lead to increased PKA activity, as has been found in the p.Ser9Asn mutation of PRKAR1A.[12] PRKAR1B knock-out mice have impaired long-term potentiation and long-term depression in the mossy fibers – Cornu Ammonis 3 region of the hippocampus and visual cortex.[42-44]

A second pathophysiological mechanism is that the Leu50Arg mutation on the subunit interface of the D/D-domain can induce structural changes to the docking site for AKAP which is located across this interface, and abolish the binding between AKAP and the regulatory subunit.[39, 40] An important function of AKAP is to target the holoenzyme in close proximity to the dedicated substrates by binding to the D/D-domain,[39] which is important for creating the microenvironment for PKA signaling. It is likely that impaired binding to AKAP causes subcellular dislocalization of the complex, thereby disturbing PKA signaling on the dedicated substrates.

The Leu50Arg *PRKAR1B* mutation and subsequent change in PKA function probably leads to an imbalance of the phosphorylation status of N-terminal head and C-terminal tail domain of neurofilaments. PKA is responsible for transient phosphorylation of specific sites on the N-terminal head domain of neurofilaments.[7] Phosphorylation levels of N- and C-terminal are related to each other, and are essential for axonal transport.[5, 7] Therefore, the imbalance of phosphorylation would explain the additional accumulation of α-internexin and other neurofilaments in the cell soma in the present cases.

The aggregate formation of PRKAR1B, its biochemical enrichment and additional bands in urea fractions can be the result of posttranslational modifications such as (hyper)phosphorylation. While our dephosphorylation experiments are not indicative for abnormal phosphorylation, more sophisticated biochemical analyses are needed to further address this. In addition, the high Mr smear in the immunoblot might be explained by poly-ubiquitination of PRKAR1B protein in the proteosomal degradation.[45] The aggregation of PRKAR1B might entrap other proteins like IFs just as a secondary phenomenon, like we see in inclusions characteristic for other neurodegenerative diseases.[1]

Although we have not seen obvious changes and presence of abnormal M_r α-internexin protein species in our cases by immunoblot, the analysis of phosphorylation alterations of IFs requires further investigation by more sensitive means such as mass spectrometry. Future studies to explore the phosphorylation of IF in model systems carrying the Leu50Arg mutation are required to address this. Finally, the unique pathological phenotype of the present disorder is supported by the distinct pattern of PKA type I regulatory bands on Western blots of brain tissue. To find out the reason of the different pattern between cases and controls, more sophisticated proteomic analysis would be required in the future.

In conclusion, we provide evidence that a mutation in *PRKAR1B* is associated with a new type of a familial neurodegenerative disease with dementia and parkinsonism characterized by specific and abundant accumulation of PRKAR1B into neuronal inclusions. Our findings link altered regulation of PKA by mutant PRKAR1B to human late-onset neurodegeneration.

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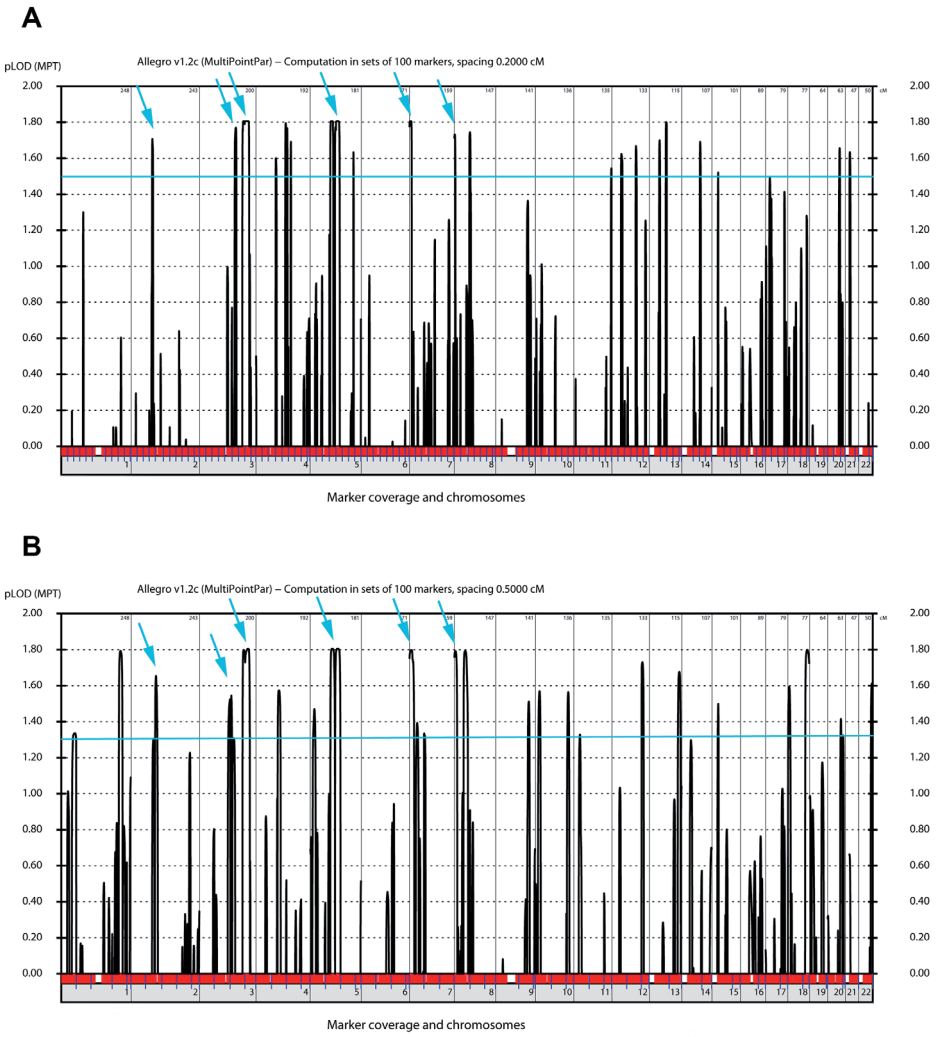
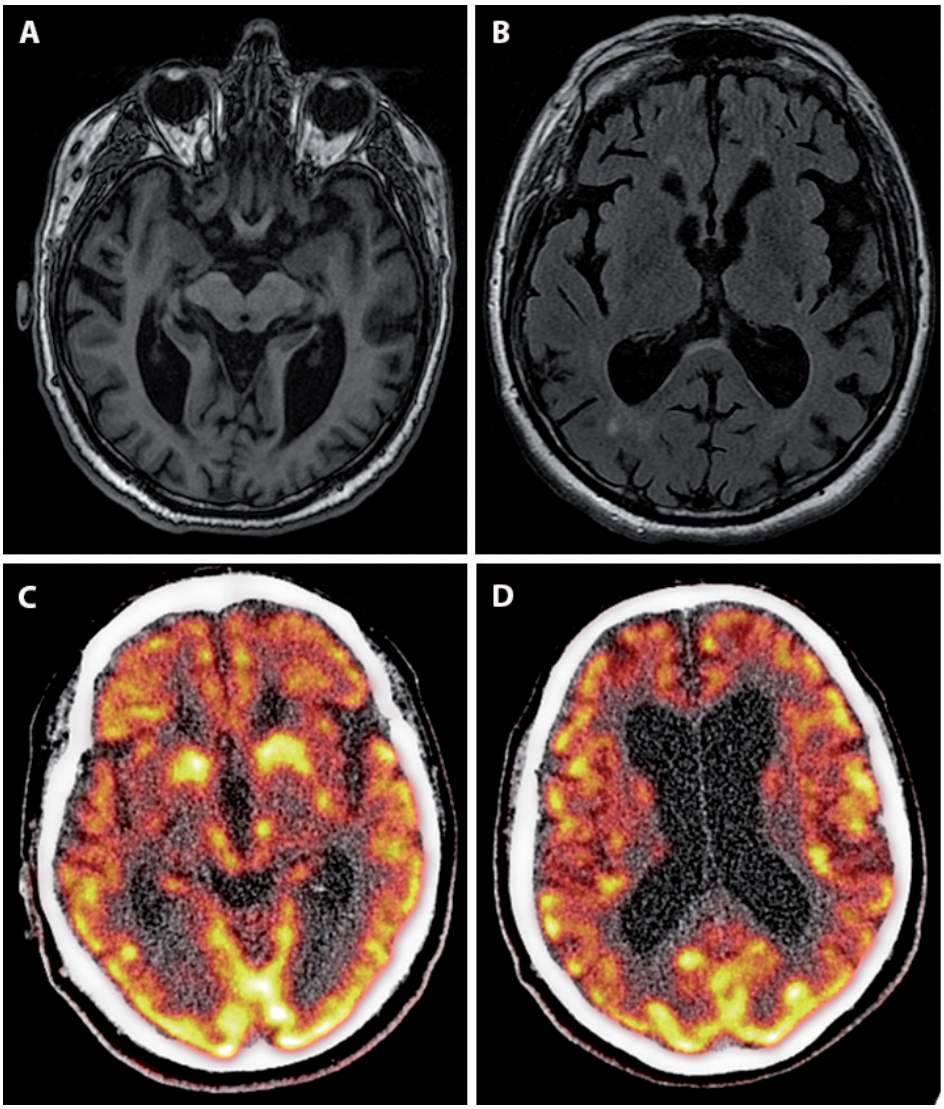
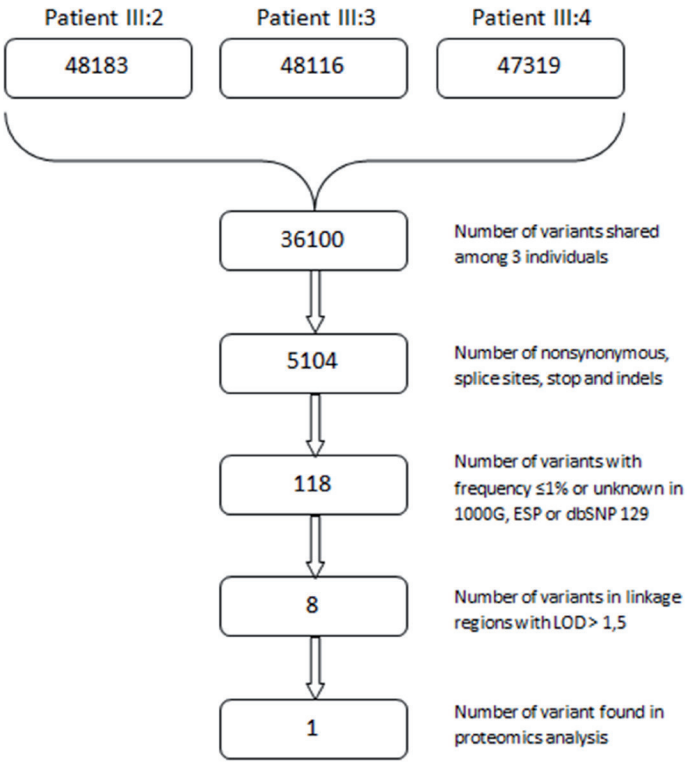
Supplementary figures and tables



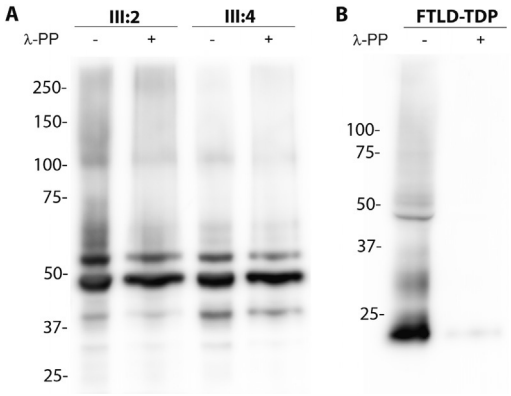
Supplementary figure 1. Plots of the LOD scores using Allegro. Two separate multipoint linkage analyses were performed (affected only) on genotypes from five affected individuals using Allegro with a SNP spacing of 0.2 cM **(A)** and one of 0.5 cM **(B)**. Regions with LOD scores >1.5 (blue line) in both models were used as candidate regions (blue arrows).



Supplementary figure 2. Neuroimaging findings from the proband (III:4). Axial images using FLAIR sequence showed symmetrical cortical atrophy and enlarged ventricles. Thinning of the mesencephalon **(A)** and atrophy of the caudate nucleus were observed **(B)**. Imaging of the brain metabolic activity using [18F] fluorodeoxyglucose and PET showed symmetrical frontal hypometabolism **(C and D)**. Metabolic deficient is also seen at the thalamus **(C)**.



Supplementary figure 3. Bioinformatic analysis of exome sequencing.



Supplementary figure 2. Biochemical analysis of phosphorylation state of PRKAR1B. **(A)** Dialyzed urea fractions from two cases with *PRKAR1B* mutation were either not treated (-) or treated (+) with lambda protein phosphatase (λ-PP), separated by SDS-PAGE and analyzed by immunoblot with anti-PKARI antibody (BD Laboratories). No obvious changes were observed in the banding pattern after dephosphorylation. **(B)** To control for de-phosphorylation activity of the used enzyme and conditions, a urea fraction from a FTLD-TDP cases was used and analyzed by immunoblot with a phosphorylation-specific TDP-43 antibody (clone 1D3). Note the lack of immunoreactivity after λ-PP treatment.

Supplementary Table 1. PCR primers and protocol for the amplification and sequencing of *PRKAR1B* fragments

Oligoname[a]	Sequence	Size
prkar1b-ex2F	GGGCCGTCACGTTTAACACC	465 bp
prkar1b-ex2R	ACCACGGGACAGAGGGAAGG	
prkar1b-ex2SF	GAGCCTGAAGGGCTGTGAGC	183 bp
prkar1b-ex2SR	AGGACACGTGCGAAGGGAAG	
prkar1b-ex3F	AAGTGGGGATGATGGGGATG	557 bp
prkar1b-ex3R	CTGAGACCCCCAGGAGGATG	
prkar1b-ex4F	TAACAGCAGGCTGAGGGTGGA	350 bp
prkar1b-ex4R	CCGGAGAAGGCAGCTGTGAT	
prkar1b-ex5F	CTGTGAAATGAGGGGAGGAAGG	436 bp
prkar1b-ex5R	CCCAGGTTCAAGCGATTCTCC	
prkar1b-ex6F	AATTGGTAGCACCCAGGATGTTG	378bp
prkar1b-ex6R	CATCACCCTTGTTTCCCTCTGC	
prkar1b-ex7F	CTCTGCCCACAAGCGAAAGG	460 bp
prkar1b-ex7R	CCTCCACCCCTTTCCACTCC	
prkar1b-ex8F	AGCTCCCTGCCCTTCATGG	529 bp
prkar1b-ex8R	TCCATAATACCAACAACACTCAACTGC	
prkar1b-ex9F	TGTCTTGGACTGTGGCTGTGG	381 bp
prkar1b-ex9R	GGGCAGGAGGAATCTCAGTGG	
prkar1b-ex10F	ACGTGGTGTCGGCAGTGG	396 bp
prkar1b-ex10R	TTGGGGGAACAGGACTGAGC	
prkar1b-ex11F	CAGGACAATGGCTAGCTGAACG	504 bp
prkar1b-ex11R	GGCCCACACCTCACACAGC	

[a] primers used for PCR amplification and Sanger sequencing reactions.

PCR protocol

The amplification reactions were performed in a total volume of 20 µl, containing 1x FastStart Taq DNA Polymerase buffer, 200 µM of each dNTP, 10 µM forward primer, 10 µM reverse primer, 0.5 unit FastStart Taq DNA Polymerase (Roche Diagnostics) and 20 ng genomic DNA. PRKAR1B_2F/R was amplified with addition of 1XGC-melt (Roche Diagnostics). PRKAR1B-ex2SF and PRKAR1B-ex2SR were used to validate the variant in sample III:9 isolated from DNA extracted from the spleen.

The PCR conditions were as follows: initial denaturation, 7 min 30 sec at 96ºC, followed by 9 cycles of: 30 sec denaturation at 96^{0}C; 30 sec annealing (1st cycle at 70^{0}C, with 1^{0}C/cycle decrease); 1 min extension at 72^{0}C. Then, 25 cycles of: 30 sec denaturation at 96^{0}C; 30 sec annealing at 60^{0}C; 1 min extension at 72^{0}C. Final extension: 5 min at 72^{0}C. The PCR reactions were purified using 5 units ExoI (Fermentas) and 1 unit Fast AP (Fermentas), 45' 37oC, 15' 80^{0}C. Direct sequencing was performed using Big Dye Terminator chemistry ver. 3.1 (Applied Biosystems) as recommended by the manufacturer. Dye terminators were removed using SephadexG50 (GE Healthcare) and loaded on an ABI 3130XL Genetic Analyzer (Applied Biosystems). For sequence analysis the software packages Sequence Analysis version 5.3 (Applid Biosystems) and Seqscape version 2.6 (Applied Biosystems) were used.

Supplementary Table 2. Candidate regions from linkage analysis

UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) Assembly		
Chromosome: start-end	**SNP: start-end**	**Size (bp)**
Chr2:72013658-95537000	rs898238-rs10165220	23523342
chr3:97749272-129292001	rs1498646-rs2244708	31542729
chr3:149604549-178635542	rs9815364-rs11715386	29030993
chr5:63649860-107594079	rs6449720-rs11240960	43944219
chr7:45989-16995059	rs6583338-rs13237658	16949070
chr8:176568-10625104	rs2003497-rs13270447	10448536

Supplementary Table 3. Exome sequencing statistic per sample

	III: 2	**III:3**	**III:4**	**Average**
Total reads	6776289400	6615714400	6449885600	6613963133
Total aligned reads	3975374358	3684889797	3408320419	3689528191
% aligned	58.66594715	55.6990459	52.84311429	55,736
% of 1X	95.7	96.2	96.1	96
% of 5X	89.7	90.2	90.8	90,23
% of 10X	83.3	84.1	85.4	84,27
% of 20X	71.7	74.2	73.9	73,24
Coverage	61.38	56.89	52.62	56,96

Supplementary Table 4. Proteomics analysis of inclusion body and controls

Protein names	Gene names	Control_1	Control_2a	Control_2b	Inclusion_1	Inclusion_2a	Inclusion_2b
Polyubiquitin-C	UBC; UBB; RPS27A; UBA52	0	0	0	39707000	123480000	23422000
Alpha-internexin	INA	0	0	0	15435000	54174000	34966000
Neurofilament light polypeptide	NEFL	0	0	0	11232000	47581000	32901000
cAMP-dependent protein kinase type I-beta regulatory subunit	PRKAR1B	0	0	0	3415200	11101000	407070
Neurofilament medium polypeptide	NEFM	0	0	0	1289300	12187000	9401100
Heat shock cognate 71 kDa protein	HSPA8; HSPA2; HSPA1B; SPA6; HSPA1A; HSPA1L; HSPA7	0	0	0	299830	671950	214140

The values represent the MS1 peptide intensities generated by Maxquant. Samples were collected twice from control_2 and Inclusion_2 and were analyzed separately as control 2a and 2b versus Inclusion 2a and 2b, respectively. The protein isoforms are clustered.

Supplementary Table 5. *In silico* prediction of functional effects of variants

		PRKAR1B p.Leu50Arg	PRKAR1B p.Arg232Gln	PRKAR1B p.Ile40Phe
PolyPhen-2	HumDiv[a]	Probably Damaging	Probably Damaging	Benign
	HumVar[b]	Possibly Damaging	Possibly Damaging	Benign
SIFT[c]		Damaging (score=0,001)	Damaging (score=0,001)	Tolerated (0,878)
PROVEAN		Deleterious	Deleterious	Neutral
MUTATION TASTER		Disease causing	Disease causing	Disease causing

[a] Preferred model for evaluating rare alleles, dense mapping of regions identified by GWAS and analysis of natural selection.
[b] Preferred model for the evaluation of Mendelian disease-causing variants, which requires distinguishing mutations with drastic effects from the remaining human variation, including abundant mildly deleterious alleles.
[c] The substitution is predicted to be damaging if the score is ≤ 0.05, and tolerated if the score is > 0.05.

Supplementary Table 6. *PRKAR1B*[a] variants detected in patients with frontotemporal dementia and Parkinson's disease

Function	dbSNP[b]	Nucleotide change	Protein change	Freq FTD[c] *n*=56	Freq PD[c] *n*=138	Freq dbSNP[d]	Freq EVS[e]
Exon 11	rs28488947	c.1065C>T	p.Phe355=	0,0000	0,0070	0,0470	A=154/G=12778
Exon 11	rs11545042	c.1014T>C	p.Thr338=	0,1786	0,0620	0,1730	G=1852/A=11016
Exon 11	rs370829885	c.1008G>A	p.Ala336=	0,0714	0,0000	-	T=11/C=12851
Exon 11	rs28626752	c.984A>G	p.Ala328	0,0000	0,0070	0,0520	C=220/T=12678
intron 10	rs28585978	c.974-8A>C		0,0000	0,0070	0,0510	G=130/T=12716
Exon 10	rs78260651	c.903C>T	p.Ser301=	0,0000	0,0040	0,0010	A=21/G=12561
Intron 9	rs71518309	c.892-18G>C		0,4196	0,4750	0,3950	G=7290/C=5128
Intron 9	rs118004775	c.892-39G>A		0,0804	0,0000	0,0720	T=863/C=11311
Intron 9	rs62431411	c.891+38A>G		0,4554	0,3010	0,3910	C=5499/T=7499
Intron 9	rs62431412	c.891+24C>T		0,5000	0,3010	0,3910	A=5498/G=7500
Exon 9	rs3211362	c.846T>C	p.Ile282=	0,5000	0,3040	0,4010	G=5629/A=7369
Exon 9	rs77809618	c.810G>A	p.Ala270=	0,0000	0,0040	0,0010	T=12/C=12986
Intron 7	rs9330368	c.709-16A>C		0,5000	0,3480	0,2810	G=8773/T=4225
Exon 7	rs200069843	c.695G>A	**p.Arg232Gln**	0,0000	0,0040	0,0010	-
Exon 7	rs76061469	c.642C>T	p.Thr214=	0,0000	0,0110	0,0010	A=22/G=12976
Intron 4	rs117395529	c.440+34G>A		0,0179	0,0000	0,0270	T=71/C=12935
Intron 4	rs142693952	c.440+25G>A		0,0000	0,0250	0,0080	T=172/C=12834
Exon 2	rs61732492	c.118A>G	**p.Ile40Phe**	0,0000	0,0220	0,0130	C=258/T=12748

[a] References for annotation of variants: GenBank n. NM_002735.2 and NP_002726.1.
[b] SNP reference number in dbSNP137.
[c] Minor allele frequency in frontotemporal dementia (FTD) and Parkinson's disease (PD).
[d] Minor allele frequency in dbSNP.
[e] Minor allele frequency in Exome variant Server.

Letter to the Editor

Reply: *PRKAR1B* mutations are a rare cause of fused in sarcoma negative neuronal intermediate filament inclusion disease

Tsz Hang Wong, Annemieke J.M.H. Verkerk, Annemieke J. Rozemuller, Rob Willemsen, Manuela Neumann, Vincenzo Bonifati, John van Swieten

We thank Dr. Pottier and colleagues for their interest in our recent study, reporting the identification of a novel mutation in the *PRKAR1B* gene in a family with FUS-negative NIFID cases.[1] Mutations in this gene seems to be rare in most neurodegenerative disorder as no additional pathogenic mutations have been found so far in patients with Parkinson's disease, frontotemporal dementia (FTD) and Alzheimer's disease.[1,2]

In the Letter, Dr. Pottier and colleagues reported the absence of mutations in *PRKAR1B* gene in small series of three pathologically confirmed NIFID cases who were FUS negative. This observation in NIFID cases with strong accumulation of neurofilament proteins is especially important, as the protein kinase A (PKA) holoenzyme has an strong effect on phosphorylation of neurofilament proteins. Therefore, we agree with their suggestion that genetic screening in the PRKAR1B pathway may identify new genetic defects in the etiology of FUS-negative NIFID. Finally, screening in *PRKAR1B* gene is still indicated in patients with frontotemporal dementia and parkinsonism, especially in those with positive family history.

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CHAPTER 3.4

3.4

Mutation frequency of *PRKAR1B* and the major familial dementia genes in a Dutch early onset dementia cohort

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Abstract

Genetic factors are important in all forms of dementia, especially in early onset dementia. The frequency of major gene defects in dementia has not been investigated in the Netherlands. Furthermore, whether the recently in a FTD family identified *PRKAR1B* gene is associated with an Alzheimer's disease (AD) like phenotype, has not been studied. With this study, we aimed to investigate the mutation frequency of the major AD and FTD genes and the *PRKAR1B* gene in a well-defined Dutch cohort of patients with early onset dementia. Mutation analysis of the genes *PSEN1*, *APP*, *MAPT*, *GRN*, *C9orf72* and *PRKAR1B* was performed on DNA of 229 patients with the clinical diagnosis AD and 74 patients with the clinical diagnosis FTD below the age of 70 years. *PSEN1* and *APP* mutations were found in, respectively 3.5 and 0.4% of AD patients, and none in FTD patients. *C9orf72* repeat expansions were present in 0.4% of AD and in 9.9% of FTD patients, whereas *MAPT* and *GRN* mutations both were present in 0.4% in AD patients, and in 1.4% resp. 2.7% in FTD patients. We did not find any pathogenic mutations in the *PRKAR1B* gene. *PSEN1* mutations are the most common genetic cause in Dutch AD patients, whereas *MAPT* and *GRN* mutations were found in less than 5 percent. *C9orf72* repeat expansions were the most common genetic defect in FTD patients. No pathogenic *PRKAR1B* mutations were found in the early onset AD and FTD patients of our study.

Introduction

Dementia is a major health problem with a world-wide prevalence of 44 million in 2013, estimated to increase to 135 million in 2050.[1] Only a minority of the patients with dementia have an early onset, with a prevalence of dementia of 1–10 in 10,000 in persons aged 45–65 years.[2] Early onset dementia is usually defined as dementia with an onset before the age of 65 years, with Alzheimer's disease (AD) and frontotemporal dementia (FTD) as most common subtypes.[2,3]

Genetic factors play an important role in all types of dementia, especially in early onset dementia. An autosomal dominant family history is found in 10–20% of the patients with early onset dementia and can be explained by mutations in the *presenilin 1* and *2* (*PSEN1* and *PSEN2*) and *amyloid-beta-protein precursor* (*APP*) genes in up to 50% of the early onset AD families, and in the *microtubule-associated-protein-tau* (*MAPT*) gene, *(pro) granulin* (*GRN*) gene and *C9orf72* gene in most FTD families.[4] The frequency of the latter gene defects in Dutch FTD patients has been reported in previous studies,[5,6] which also showed a wide variation in the clinical presentation in mutation carriers, including an AD-like presentation. In contrast, mutation screening has not been carried out in Dutch early onset AD patients so far.

Recently, a mutation in the *PRKAR1B* gene has been identified in a Dutch family with an autosomal dominant FTD-like presentation. Mutations in this gene are probably a rare cause of FTD, as no pathogenic variants were found in other patients with familial FTD7. Its phenotype encompasses behavioural and cognitive changes with mild parkinsonism. However, no pathogenic mutations were found in a cohort of familial Parkinson patients.[7] Whether mutations in the *PRKAR1B* gene may result in an AD-like phenotype, as is the case for *GRN* and some specific *MAPT* mutations, has not been studied.

With this study, we aimed to estimate the mutation frequency of the most common autosomal dominant AD and FTD genes in Dutch early onset AD and FTD patients, and to investigate whether mutations in the newly identified *PRKAR1B* gene are a frequent cause of early onset AD.

Patients and methods

Subjects

We included all patients with a clinical diagnosis FTD (*n*=74) or AD (*n*=229) below the age of 70 years from the memory clinic-based Amsterdam Dementia Cohort who visited the Alzheimer centre of the VU University Medical Centre in Amsterdam between October 2000 and November 2008 of whom DNA was available for research purposes.

All patients were evaluated following a standard protocol including neurological examination, neuropsychological testing, biochemical analysis of blood, neuroimaging, and EEG. The clinical diagnosis was made by consensus in a multidisciplinary team. The diagnosis "possible" or "probable" AD was made according to the NINCDS-ADRDA criteria,[8] FTD according to the criteria of Neary et al.[9] In all subjects with possible or probable AD, cerebrospinal fluid (CSF) was collected for scientific purposes, however, the CSF results were not included in the clinical decision making. Our laboratory applies reference values of $A\beta_{1\text{-}42} \leq 550$ pg/ml and t-tau > 375 pg/ml.[10] The age at onset was defined as the age at which the first symptom, compatible with cognitive decline or with the diagnosis FTD, was observed by the spouse or a close relative. Family history was obtained from the medical records and considered positive in case of at least one 1st degree with dementia or, in FTD patients, with dementia or amyotrophic lateral sclerosis (ALS). Autosomal dominant inheritance was defined as the occurrence of at least three affected persons in at least two generations of one family. The characteristics of the patients can be found in Table 1. Of the patients with FTD, 68 were also included in our previous study on the mutation frequency of *C9orf72* repeat expansions.[6]

Table 1. Characteristics of the patients

	FTD patients (*n*=74)	AD patients (*n*=229)
Male (percentage)	45 (60.8)	104 (45.4)
Mean age at diagnosis, years (range)	61.2 (46–69)	61.0 (35–69)
Mean disease duration at diagnosis (range)	3.9 (0–18)	3.5 (0–13)
Mean age at death, years (range)*	63.2 (46–71)	63.9 (39–74)
Positive family history (percentage)**	28 (37.8)	80 (34.9)

* Information on age at death available of 24 FTD and 47 AD patients.
** Positive family history in case of at least one first degree relative with dementia or, in FTD patients, with ALS.

Genetic analysis

Genomic DNA was extracted from peripheral-blood leukocytes according to standard procedures. All exons and exon–intron boundaries of the genes *PSEN1*, *MAPT*, *GRN* and *PRKAR1B* and exons 16 and 17 of the *APP* gene were amplified from genomic DNA (conditions and primer sequences available upon request). Direct sequencing was performed using BigDye terminator chemistry (Applied Biosystems, Foster City, CA), and sequencing products were processed on an Applied Biosystems 3730 automated DNA sequencer and analysed using SeqScape software version 2.7 (Applied Biosystems). Nucleotides are numbered according to Genbank accession numbers NM_007318 (*PSEN1*), NM_201414 (*APP*), NM_005910 (*MAPT*), NM_002087 (*GRN*) and NM_002735 (*PRKAR1B*) with A of initiator ATG numbered as +1.

In 181 AD patients, analysis of the *PRKAR1B* gene was performed on exome sequencing data. Whole exome capture and sequencing were performed by Human Genomics Facility at Rotterdam. Exomes were captured by Nimblegen seqcap EZ human exome v3, and were sequenced with 2 × 100 paired-end sequencing on the Illumina HiSeq 2000 platform, according to the manufacturer's protocol. Reads were mapped to the human reference genome sequence (UCSC hg19) using the Burrows-Wheeler Alignment Tool.[11] Duplicate read removal, local sequence realignment and variant filtering to minimize base calling and mapping errors were performed by Samtools,[12] Picard (http://picard.sourceforge.net) and Genome analysis Tool Kit (GATK).[13] The identified variants per individual were called using GATK and annotated by ANNOVAR.[14] Variants with quality score < 50, quality over depth < 5.0, Strand bias > 0.75 and depth < 5.0 were filtered out. The average read depth for the *PRKAR1B* gene was 402×, with a range of 11 (exon 2) to 832. Variants in the *PRKAR1B* were examined on their frequency in dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP, build 138), the 1,000 genome project (www.1000genomes.org), the National Heart Lung Blood Institute Exome Variant Server (EVS) (https://evs.gs.washington.edu/EVS) and the Genome of the Netherlands (GoNL) (http://www.nlgenome.nl). Predicted functional effects of all protein-coding PRKAR1B were assessed by Polyphen-2 (http://genetics.bwh.harvard.edu/pph2), Sorting Intolerant from Tolerant (SIFT) (http://sift.jcvi.org/www/SIFT_enst_submit.html), PROVEAN (http://provean.jcvi.org/seq_submit.php) and Mutation Taster (www.mutationtaster.org). Splice site prediction was performed by Alamut 2.0 (Interactive Biosoftware). Possible pathogenic variants were confirmed by Sanger sequencing.

Patients were screened for *APP* gene deletions and duplications using the multiplex ligation-dependent probe amplification (MLPA) method and SALSA kit P170 *APP* probes mix according to the manufacturer's instructions (MRC-Holland b.v.) Results were analyzed using GeneMarker Software (version 1.75, SofGenetics, LLC). Dosage ratio values of ≤ 0.7 and ≥ 1.3 were used as boundaries for deletions and duplications, respectively. At least one negative control was used, no positive controls were available.

The hexanucleotide repeat expansion in the *C9orf72* gene was determined by repeat-primed polymerase chain reaction as described previously.[6,15] A cut-off value of 30 repeats was used to define expanded repeats.[16] The *C9orf72* analysis was successful in 218 AD and 72 FTD patients.

Statistical analysis

Means and percentages were calculated with SPSS, version 20.0 (Chicago, Ill., USA).

Table 2. Variants detected in the PRKAR1B gene[a]

Location	Nucleotide change	Protein change	dbSNP[b]	FreqFTD[c]	FreqAD[c]	MAF dbSNP[d]	MAF EVS[e]	MAFGoNL[f]
Exon 2	c.6C>T	p.Ala2Ala	-	0.0000	0.0023	-	-	-
Exon 3	c.254C>A	p.Thr85Asn	-	0.0082	0.0000	-	-	-
Exon 4	c.415G>A	p.Ala139Thr	rs185641179	0.0000	0.0023	0.0005	-	0.0010
Exon 5	c.477C>T	p.Ile159Ile	-	0.0000	0.0023	-	-	-
Exon7	c.642C>T	p.Thr214Thr	rs76061469	0.0000	0.0023	0.0009	0.0017	0.0030
Intron 7	c.708+6T>C	n/a	rs74939612	0.0000	0.0023	0.0018	0.0017	0.0060
Exon 9	c.810G>A	p.Ala270Ala	rs77809618	0.0000	0.0023	0.0009	0.0009	0.0010

[a] Exonic and splice site variants in the PRKAR1B-gene with a MAF <0.01 (dbSNP). References for annotation of variants: GenBank n. NM_002735.2 and NP_002726.1.
[b] SNP reference number in dbSNP137.
[c] Minor allele frequency in our cohort of AD and FTD patients.
[d] Minor allele frequency in dbSNP.
[e] Minor allele frequency in the National Heart Lung Blood Institute Exome Variant Server (EVS).
[f] Minor allele frequency in Genome of the Netherlands (GoNL).

Results

No pathogenic *PRKAR1B* mutations were identified in AD or FTD patients. Nonetheless, several variants were found, mainly synonymous variants (Table 2). In two AD patients, a non-synonymous variant of unknown significance was found. The variants p.Thr85Asn and p.Ala139Thr are predicted to be benign by at least 3 out of 4 software programs. The variant c.708+6T>C, possibly affecting a splice site, was found in one AD patient. This variant is a known SNP with a prevalence of 0.6% in the Dutch population and predicted to be benign by 4 out of 5 splice site prediction algorithms, and therefore, unlikely to be pathogenic.

Mutations in the *PSEN1* gene were found in 8 out of the 229 patients with AD (3.5%), and a duplication of the *APP* gene, a *MAPT* mutation, a *GRN* mutation and a *C9orf72* repeat expansion were each identified in one AD patient (0.4%). All mutations found in AD patients have been described previously and are considered to be pathogenic (www.molgen.vib-ua.be/ADMutations). The mutations and a description of the presenting phenotype of the patients can be found in Table 3. The overall mutation frequency in AD patients was 11% in familial and 2% in sporadic cases. The parents of the sporadic patients with *PSEN1* mutations died at an age older than 64 years. Information on the current age or age of death of the parents of the patient with the *C9orf72* repeat expansion is lacking. The CSF analysis in the patients with an AD-like clinical presentation and a *MAPT* mutation or *C9orf72* repeat expansion showed a normal amyloid-beta and increased tau level, while increased tau and decreased amyloid-beta levels were found in the patient with the AD-like presentation and the *GRN* mutation.

Analysis of 74 FTD patients showed one *MAPT* mutation (1.4%), two *GRN* mutations (2.7%), seven *C9orf72* hexanucleotide repeat expansions (9.9%) and no mutations in the genes *PSEN1* and *APP*. A description of the presenting phenotype of the patients with mutations can be found in Table 4. The p.Asn296Asp mutation in the exon 10 of *MAPT* gene is likely to be pathogenic as several other pathogenic mutations have been reported involving this amino acid, although this mutation has not been reported earlier. Both mutations in *GRN* gene have been described before as pathogenic mutations (www.molgen.vib-ua.be/ADMutations). All FTD patients with mutations had a positive family history for dementia or ALS, the mutation frequency in familial cases was, therefore 36%, and zero in sporadic cases.

Table 3. Characteristics of AD patients with a genetic defect

Case	Gene	Nucleotide change	Amino acid change	Age at onset	Age at diagnosis	Age at death	Family history	Presenting phenotype	Imaging at presentation	CSF analysis (pg/ml)	
AD01	*PSEN1*	c.236C>T	p.Ala79Val	53	57	64	Positive	Memory loss, mild executive function and language disorders, apathy	Parietal atrophy, no hippocampal atrophy	$A\beta_{1-42}$ Tau	418 (↓) 836 (↑)
AD02	*PSEN1*	c.236C>T	p.Ala79Val	51	53	57	Positive	Memory loss, executive and visuo-spatial function disorders	Global atrophy including the hippocampi	$A\beta_{1-42}$ Tau	175 (↓) 443 (↑)
AD03	*PSEN1*	c.344A>G	p.Tyr115Cys	40	41	45	Autosomal dominant	Memory loss, executive and visuo-spatial function disorders	Parietal atrophy, no hippocampal atrophy	$A\beta_{1-42}$ Tau	377 (↓) 432 (↑)
AD04	*PSEN1*	c.367G>A	p.Glu123Lys	64	67	70	Negative	Memory loss, executive and visuo-spatial function disorders, language disorders	Global cortical atrophy	$A\beta_{1-42}$ Tau	487 (↓) 491 (↑)
AD05	*PSEN1*	c.617G>A	p.Gly206Asp	33	35	39	Autosomal dominant	Memory loss, executive and visuo-spatial function disorders, language disorders	Global cortical atrophy	$A\beta_{1-42}$ Tau	452 (↓) 1647 (↑)
AD06	*PSEN1*	c.806G>A	p.Arg269His	66	68	n/a	Negative	Memory loss and language disorders	Mild parietal and hippocampal atrophy, vascular white matter disease	$A\beta_{1-42}$ Tau	337 (↓) 622 (↑)

Table 3. *Continued*

Case	Gene	Nucleotide change	Amino acid change	Age at onset	Age at diagnosis	Age at death	Family history	Presenting phenotype	Imaging at presentation	CSF analysis (pg/ml)	
AD07	*PSEN1*	c.806G>A	p.Arg269His	57	62	n/a	Positive	Memory loss, executive and visuo-spatial function disorders	Global, predominantly parietal atrophy, no hippocampal atrophy	$A\beta_{1-42}$ Tau	378 (↓) 3150 (↑)
AD08	*PSEN1*	c.1130G>T	p.Arg377Met	51	56	n/a	Positive	Memory loss	Parietal atrophy	$A\beta_{1-42}$ Tau	311 (↓) 519 (↑)
AD09	*APP*	Whole gene duplication	n/a	47	52	59	Positive	Memory loss, executive function disorders	Normal	$A\beta_{1-42}$ Tau	359 (↓) 594 (↑)
AD10	*GRN*	c.388_391 delCAGT	p.Gln130fs	42	47	n/a	Positive	Memory loss, apathy, mild language disorder	Parietal atrophy, no hippocampal atrophy	$A\beta_{1-42}$ Tau	477 (↓) 573 (↑)
AD11	*MAPT*	c.2221C>T	p.Arg406Trp	55	59	n/a	Autosomal dominant	Memory loss	Hippocampal atrophy	$A\beta_{1-42}$ Tau	1026 442 (↑)
AD12	*C9orf72*	Repeat expansion	n/a	64	69	70	Negative	Memory loss, executive function disorders, mild visuo-spatial function disorders, apathy, disinhibition	Frontal and medial temporal atrophy, mild cerebellar atrophy	$A\beta_{1-42}$ Tau	945 607 (↑)

n/a: not applicable

Table 4. Characteristics of FTD patients with a genetic defect

Case	Gene	Nucleotide change	Amino acid change*	Age at onset	Age at diagnosis	Age at death	Family history for dementia	Family history for ALS	Presenting phenotype	Imaging at presentation
F01	*MAPT*	c.886A>G	p.Asn296Asp	49	62	62	Autosomal dominant	Negative	Disinhibition, stereotype / compulsive behaviour, language disorders, memory loss	Temporal atrophy
F02	*GRN*	c.380_381 delCT	p.Pro127fs	48	52	56	Positive	Negative	Apathy, executive function disorders	Frontal and temporal atrophy
F03	*GRN*	c.388_391 delCAGT	p.Gln130fs	56	59	n/a	Positive	Negative	Memory loss, mild executive function disorders, apathy	Right temporal atrophy
F04	*C9orf72*	Repeat expansion	n/a	60	61	61	Positive	Negative	Language disorders, mild disinhibition	Asymmetric frontal and temporal atrophy
F05	*C9orf72*	Repeat expansion	n/a	61	64	70	Autosomal dominant	Positive	Apathy, language disorders	Global atrophy
F06	*C9orf72*	Repeat expansion	n/a	55	65	60	Negative	Positive	Disinhibition, mild stereotype / compulsive behaviour, language disorders, mild memory loss, mild visuo-spatial function disorders	Normal

Table 4. *Continued*

Case	Gene	Nucleotide change	Amino acid change*	Age at onset	Age at diagnosis	Age at death	Family history for dementia	Family history for ALS	Presenting phenotype	Imaging at presentation
F07	*C9orf72*	Repeat expansion	n/a	49	59	64	Positive	Positive	Disinhibition, mild stereotype / compulsive behaviour, memory loss	Normal
F08	*C9orf72*	Repeat expansion	n/a	41	46	46	Positive	Negative	Apathy, language disorders, memory loss, executive and visuo-spatial function disorders	Global atrophy
F09	*C9orf72*	Repeat expansion	n/a	58	61	64	Positive	Negative	Mutism, apathy, stereotype / compulsive behaviour, hallucinations	Global atrophy
F10	*C9orf72*	Repeat expansion	n/a	50	55	60	Autosomal dominant	Negative	Disinhibition, apathy, mild stereotype / compulsive behaviour	Frontal atrophy

n/a: not applicable

Discussion

The main finding of our study is that we found no evidence for pathogenic *PRKAR1B* mutations in a large cohort of patients with early onset dementia. We found an overall mutation frequency of 5% in AD patients, with *PSEN1* being the most commonly mutated gene and a low frequency of mutations in other genes. In FTD patients, the mutation frequency was 14%, mostly consisting of hexanucleotide repeat expansions in the *C9orf72* gene. We identified *MAPT* and *GRN* gene mutations and a repeat expansion in the *C9orf72* gene in three patients with clinically AD.

The negative results of the *PRKAR1B* analysis suggest that mutations in this gene are rare in early onset dementia patients. Although it is likely that mutations in this gene are not associated with an AD-like phenotype, this needs to be studied in a larger cohort.

The observed mutation frequency of common genes in both early onset AD and FTD patients is relatively low compared to other studies.[4] Most of our FTD patients were previously included in a Dutch study, in which a total mutation frequency of 26.3% was found in FTD patients.[6] However, this latter cohort was enriched by a large proportion of patients with an autosomal dominant family

history, referred to our collaborating centre with special expertise in hereditary FTD. The frequency of *C9orf72* repeat expansions is comparable between the past and present study.

The age at onset and phenotype of the patients with mutations are in most cases in concordance with previously described associated phenotypes. Interestingly, mutations were found in three AD patients with a negative family history. Furthermore, one of the patients with the *PSEN1* mutation p.Arg269His had his first manifestations at the age of 66 years, a late onset according to the most commonly used definition. This mutation was previously found in several families and is most often associated with early onset AD.[17-19] In one family, however, the mean age at onset was above 65 years.[20] These findings suggest that a late onset of AD and a negative family history do not by definition exclude an identifiable genetic cause.

Interestingly, 13% (3/13) of the mutations in patients with the clinical diagnosis of AD were found in a FTD gene. An Alzheimer-like phenotype has been previously described in patients with the mutation p.Arg406Trp in the *MAPT* gene (www.molgen.vib-ua.be/ADMutations), and this specific case has been described before.[21] A phenotype with predominant memory loss resembling AD is frequently seen in *GRN* mutations.[22] Although low CSF amyloid-beta levels have been described in *GRN* mutation carriers,[23] a low amyloid-beta and high tau level in the CSF in this patient strongly suggests a concomitant AD. Furthermore, the clinical diagnosis frontal variant of AD in the patient with a *C9orf72* repeat expansion could retrospectively be considered as a misdiagnosis

by the presence of a normal CSF amyloid-beta and increased tau level. This is further supported by frontal, temporal and cerebellar atrophy on MRI compatible with FTD, especially a *C9orf72* repeat expansion.

One of the strengths of our study is the well-defined large cohort of early onset dementia patients. The extensive investigations performed on patients with dementia are likely to have resulted in a correct diagnosis in most patients, especially if the CSF results had been included in the clinical decision making. This is confirmed by the low frequency of mutations in the FTD genes in EOAD patients with an AD CSF profile, and no mutations in the AD genes in FTD patients.

One major limitation of our study is that we performed genetic analysis of common genes associated with AD and FTD only. As the frequency of mutations in other genes, like *PSEN2*, *CHMP2B*, *VCP* and *TDP-43*, is very low, we did not expect to find any mutations in these genes. Furthermore, the sensitivity and specificity of the method used for the analysis of the *C9orf72* repeat expansion differs by performing laboratory.[24] Since unreliable results of the *C9orf72* analysis were not included in the outcome, the actual mutation frequency may be slightly higher than reported. Also, since the depth of the exome sequencing was quite low for some exons of the *PRKAR1B* gene, it is possible that mutations in this gene have been missed.

In conclusion, no pathogenic *PRKAR1B* mutations were found in our Dutch cohort of early onset AD and FTD patients. Furthermore, mutations in AD patients are quite rare, but are occasionally also found in patients with a late onset dementia or lacking a positive family history. Therefore, patients and relatives with questions about the heritability of the dementia should be referred to a clinical geneticist for counselling independently of age at onset and family history. Also, mutations in genes associated with FTD may cause an AD-like phenotype. In FTD patients, we only found mutations in patients with a positive family history for dementia and/or ALS. However, previous studies have shown that especially repeat expansions in the *C9orf72* gene may be present in sporadic cases.[6,16] Therefore, also all FTD patients with questions about a genetic cause should be offered genetic counselling.

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CHAPTER 4

4

General discussion

During the last decade, major advances have been made in the genetics of Alzheimer's disease (AD) and frontotemporal lobar degeneration (FTLD) by the introduction of next generation sequencing (NGS) which allowed us to study rare genetic variants with large effect size on disease risk. Whole exome sequencing (WES) has also been proven to be a cost-effective approach to identify underlying genetic causes in neurodegenerative diseases, in particularly in Mendelian form of disease. Patients with previously unspecified dementia have been given a definite diagnosis. Several new genetic defects in known or novel genes have been found, and have also resulted in further classification of neurodegenerative diseases. To date, no cure is available for AD and FTLD. The confirmation of a specific genetic form of dementia is important for future drugs trials, especially for starting therapy in early stage of disease process. The advantages of WES compared to traditional Sanger sequencing has led to a rapid implementation in clinical diagnostic process. However, WES could result in many genetic variants with uncertain significance (VUS) to disease or incidental genetic findings. This have raised questions on how to characterize these findings and how to inform patients and families about these findings.

This thesis aimed to study the contribution of rare genetic variations in AD and FTLD using WES, and to describe corresponding clinical and pathological features of these mutations carriers. The main findings reported in this thesis will be outlined and discussed in relation to other studies. At last, we will provide recommendations for genetic variations identified by WES, and will discuss the future direction for genetic research in AD and FTLD and the clinical implementation of NGS.

Genetic heterogeneity in Alzheimer's disease

AD is a genetically complex disease with a highly heritable component.[1] The genetics of AD is heterogeneous encompassing highly penetrant autosomal dominant mutations usually in early-onset AD (EOAD), and genetic risk factors contributing to risk on late-onset AD. With the introduction of NGS in genetic research of AD, several new genes have been identified involving both EOAD and late-onset AD (LOAD). Rare variants in Sortilin-related receptor 1 (*SORL1*), ATP Binding Cassette Subfamily A Member 7 (*ABCA7*) and Triggering Receptor Expressed on Myeloid Cells 2 (*TREM2*) have been consistently replicated to be associated with AD, whereas the contribution of several candidate genes in AD such as Phospholipase D3 (*PLD3*) and Unc-5 netrin receptor C (*UNC5C*) remained uncertain.[2] One possible explanation for the lack of association might be phenotypic variability among patients with neurodegenerative disease, which challenges clinician to define correct clinical diagnosis.

PSEN1 and *PSEN2* in EOAD

In **Chapter 2.1** we reported three presenilin 1 (*PSEN1*) and two presenilin 2 (*PSEN2*) variants identified in a Dutch cohort of 68 EOAD patients using WES. Three variants, one in *PSEN1* and two in *PSEN2*, have not been previously reported. These novel variants, except one frameshift variant in *PSEN1* (p.H21Profs*2), were assigned as probable pathogenic according to the algorithm proposed by Guerreiro et al.[3] The pathogenicity of these three novel variants is also supported by their absence in Exome aggregation consortium database and the exome database from the Rotterdam study. However, we have classified the variant *PSEN1* p.H21Profs*2 as a VUS as clinical significance of frameshift mutations in *PSEN1* is uncertain, and supporting functional experiments and segregation data in the family were absent.[4-6]

The highly variable clinical presentation of the *PSEN1* and *PSEN2* variant carriers in our study, including behavioral changes and gait disturbances occasionally accompanied with spastic paraparesis or hallucinations, is in line with previous reported studies.[5, 7] Up to 16% of *PSEN1* mutation carriers have been reported to have an atypical initial clinical presentation,[7] which challenges the diagnostic process during lifetime. For this reason, WES could be helpful for diagnostic purposes.

EIF2AK3 associated with AD

In **Chapter 2.2**, we described eight families with a suggestive autosomal dominant mode of inheritance for AD, and absence of *PSEN1*, *PSEN2* and *APP* gene mutations. Using WES, we have identified two distinct rare variants in Eukaryotic Translation Initiation Factor 2 Alpha Kinase 3 (*EIF2AK3*) gene segregating with disease in two AD families. *EIF2AK3* encodes for protein kinase RNA-like endoplasmic reticulum kinase (PERK), which functions as a sensor in the endoplasmic reticulum involved in the activation of the unfolded protein response (UPR).[8] PERK has been implicated to be involved in learning, memory and pathogenesis of AD.[9, 10] The association of rare variants in *EIF2AK3* with AD in our study has also been supported by an enrichment of rare variants in the WES data of Dutch individuals with AD compared to healthy non-demented controls. One previous study has reported an association of one single nucleotide polymorphism in *EIF2AK3* with AD in *APOE* ε4 carriers,[11] but no studies have examined the contribution of rare variant in *EIF2AK3* with AD to our knowledge. Burden test by collapsing all variants in *EIF2AK3* with MAF < 1% showed an almost two times increased risk of AD. It has to be noted that the single p.Arg240His variant was the main driver for this association. Although there was a potential risk modifying effect of this variant for AD, we could not demonstrate this association in an independent cohort of AD cases and non-demented controls, but there was a trend towards association. The lack of significance could be due to the relatively small sample size in our study. Future replication studies with larger sample sizes are needed to investigate the association of *EIF2AK3* with AD.

Neuropathologically, we observed an increased activation of UPR markers phosphorylated (p) PERK and phosphorylated eukaryotic initiation factor-2α (peIF2α) among the *EIF2AK3* carriers compared to non-demented controls suggesting an increased UPR activity. However, this increased UPR was similar to non-*EIF2AK3* carriers with AD, implicating that these *EIF2AK3* variations may not induce more UPR activation in the pathomechanism of AD. Activated UPR has been reported in patients with AD and progressive supranuclear palsy (PSP).[12, 13] One possible explanation is that *EIF2AK3* variants may activate UPR early in the disease process, but do not differ in the end stage of the disease.

The exact mechanism how rare *EIF2AK3* variants contribute to AD is unclear. We hypothesized that *EIF2AK3* variants may enhance PERK signaling, resulting in increased phosphorylation of tau by glycogen synthase kinase 3β (GSK3β) and amyloidogenesis (by β-site APP cleaving enzyme 1 (BACE1)). Previous studies have indicated that PERK-eIF2α signaling is involved in the modulating of tau phosphorylation and amyloid precursor protein (APP) processing in AD.[9, 14, 15] Increased PERK-eIF2α signaling is also correlated with the level of tau pathology in PSP and AD.[13, 14] Interestingly, genetic and pharmacological modulation of PERK pathway in mouse model has resulted in amelioration of clinical and neuropathological features of AD and related disorders, suggesting this could be a potential therapeutic target for the treatment and prevention of neurodegeneration.[16] However, severe pancreatic side effects such as hyperglycemia and pancreatic toxicity hinder the translation of PERK signaling modifying medication to be implemented in clinical trials.

Our study further supports the genetic contribution of *EIF2AK3* to the pathogenesis of AD, and may provide new insights in the pathogenesis of AD, and hopefully in the therapeutic interventions for AD patients. Future functional studies are warranted to investigate the contribution of rare variants in *EIF2AK3* to the development of AD.

Genetic heterogeneity in frontotemporal lobar degeneration

FTLD is characterized by a highly variable clinical presentation, and can be accompanied with amyotrophic lateral sclerosis (ALS) as well as atypical parkinsonian disorder including corticobasal syndrome (CBS) and PSP.[17] At genetic level, FTLD is heterogeneous, and has a strong genetic component with positive family history among FTLD patients up to 50%.[18] Pathologically, neuronal depositions of transactive response DNA-binding protein of 43 kDa (TDP43) or tau protein can be found in majority of FTLD cases. FTLD and ALS have considerable clinical, genetic and pathological overlap, and can be classified as FTLD-ALS spectrum disorder with possibly sharing disease mechanism (Nguyen 2018).[19] Since the discovery of *C9orf72* repeat expansion as major genetic cause of FTLD-ALS spectrum, several new FTLD-ALS causing genes including Tank binding kinase 1 (*TBK1*), Coiled-coil-helix-coiled-coil-helix domain-containing protein 10 (*CHCHD10*)

and Tubulin alpha 4A (*TUBA4A*) have been identified using NGS technologies.[20, 21] In **Chapter 3.1** and **3.2** we demonstrated mutations in two ALS genes (*VCP* and *TUBA4A*) with pure FTLD phenotype. In **Chapter 3.3** and **3.4** we demonstrated a novel gene mutation causing a rare form of FTLD, and aimed to estimate the frequency of this mutation in a cohort of neurodegenerative diseases.

VCP

In **Chapter 3.1** we reported two novel and one earlier reported mutation in valosin-containing protein (*VCP*) gene in 85 patients with frontotemporal dementia (FTD), identified using WES and targeted sequencing of known causative genes involved in neurodegenerative disorders. All patients were negative screened for mutations in microtubule associated protein tau (*MAPT*), progranulin (*GRN*) and Chromosome 9 open reading frame 72 (*C9orf72*). The pathogenicity of VCP mutations was supported by: 1) previously reported mutations in the same codon; 2) absence in healthy control exomes and public exome databases;[22-26] 3) neuropathological findings consistent with FTLD-transactive response DNA-binding protein (TDP) type D.[27] A wide variation in phenotypic presentation has been reported among *VCP* mutation carriers, even within the same families.[28, 29] In our study, behavioral variant FTD (and additional semantic impairment in one) without any signs of motor neuron disease or myopathy was the main phenotype of the mutations carriers. This clinical presentation was in contrast with classical *VCP* mutation associated phenotype of inclusion body myopathy with Paget's disease of the bone and FTD,[30] albeit *VCP* mutation carriers with pure FTD or MND has been frequently reported.[23-26] Similar to previous studies, we have also observed a variable age at onset ranging from 41 to 60. This large clinical variability indicates the presence of possible genetic modifiers. However, no genetic modifiers have been found in *VCP* mutations carriers so far. Future research including large cohort of *VCP* mutation carriers with distinct phenotype may help us in the search for these (genetic) modifying factors.

TUBA4A

An enrichment of *TUBA4A* variants has initially been reported in an exome-wide analysis of familial ALS patients compared to controls, however, the evidence for its causal role in FTD and/or ALS is scarce due to: 1) limited number of replication in other FTD and/or ALS cohorts; 2) absence of cosegregation with disease.[19, 21] In **Chapter 3.2** we reported a novel *TUBA4A* variant cosegregated with disease in a family with FTD phenotype. This variant was assumed to be probably pathogenic based on a high Combined annotation-dependent depletion (CADD) score, unknown in GnomAD database, and cosegregation in five affected individuals. Behavioral variant FTD was the phenotype of three patients, and dementia with parkinsonism in one. No symptoms of ALS have been noticed. Neuropathologically, a mixed of FTLD-TDP type A and type B characteristics has been observed in one carrier.[27]

Although *TUBA4A* variants have been reported to be a rare genetic cause of ALS,[6, 21, 31] its genetic contribution in FTD remains unclear. Only one study has reported a frameshift mutation in *TUBA4A* (p.Arg64Glyfs*90) in a patient with clinical FTD phenotype without supporting neuropathology.[6] We reported for the first time a novel *TUBA4A* variant cosegregating with disease in an extended family with FTD and/ dementia including neuropathological features of one carrier, indicating that *TUBA4A* could be a rare genetic cause of FTD. Although the potential involvement of *TUBA4A* variant in our FTD family is supported by cosegregation and in silico prediction tools, we could not rule out that other candidate variants identified by WES are possibly related to the disease in this family. Functional studies are needed to investigate the potential role of this variant in the pathogenesis of FTD.

Novel *PRKAR1B* gene in frontotemporal lobar degeneration

In **Chapter 3.3** we reported a large family with dementia and parkinsonism with unique neuropathological features. The clinical phenotype is rather unspecific consisting of uncharacterized dementia, FTD and atypical parkinsonian disorder. Neuropathological examination of three patients showed mild cerebral atrophy with abundant neuronal cytoplasmatic inclusions (NCI) positive stained for p62, neurofilament, and α-internexin, but negative for fused in sarcoma (FUS), α-synuclein, and TDP43. This neuropathological phenotype is in contrast with neuronal intermediate filament inclusion disease (NIFID), in which FUS and α-internexin-IR NCI have been observed.[32, 33] By combination of genome-wide linkage analysis, WES and proteomic analysis we identified a novel mutation (p.Leu50Arg) in protein kinase A type I-beta regulatory subunit (*PRKAR1B*) gene cosegregating in the family. The unique neuropathological phenotype consisting of abundant PRKAR1B-IR NCI (which are absence in other neurodegenerative disorders), accompanying with biochemical alteration on western blot supported the pathogenic effect of this mutation.

Two possible pathomechanisms of this mutation could be hypothesized: 1) by affecting effective dimerization between the dimerization/docking (D/D) domains within the protein kinase A (PKA) holoenzyme, and as a consequence exposing hydrophobic protein regions that may result in aggregation; 2) by reducing the binding of the regulatory subunits to both the catalytic subunits of PKA, and to A anchoring protein (AKAP), a protein involved in targeting PKA signalling. In vivo and in vitro studies should be done to elucidate the exact effect of PRKAR1B on its substrate.

Our study has evidenced the success of combining NGS with proteomics techniques (proteogenomic method) to identify disease-causing gene. Interestingly, a similar approach has also been applied in one study resulting in the identification of disease-associated genes.[34] Future studies should favouring the use of combination of different omics data in genetic studies.

PRKAR1B mutation is assumed to be a rare cause of FTLD as no additional mutations are reported to date. Screening for *PRKAR1B* variants in FUS negative NIFID cases did not find any mutation in one study.[35] In **Chapter 3.4** we aimed to estimate the frequency of *PRKAR1B* in a Dutch cohort of patients with clinical diagnosis FTD (*n*=74) or AD (*n*=229) below the age of 70. We have identified seven variants in *PRKAR1B* gene, of which two nonsynonymous variants with unknown significance. Although the frequencies of both variants were rare (< 1%) in public database such as Exome Variant Server and Genome of the Netherlands, these variants were predicted to be benign by most in silico prediction tools. Neither neuropathology available nor cosegregation analysis supporting the pathogenic nature of these variants were available.

The identification of *PRKAR1B* mutation in FTLD extends our understanding of genetic and neuropathology of FTLD. The lack of studies reporting *PRKAR1B* mutation indicates that this mutation is a rare cause of FTLD. Large sequencing studies are essential to estimate the frequency of *PRKAR1B* mutation in FTLD and other neurodegenerative diseases. Functional studies are warranted to investigate the disease mechanism of PRKAR1B mutation and its relation with intermediate filaments.

The variability in clinical presentation complicates making the correct diagnosis, resulting in overlapping diagnosis. We have demonstrated that WES could be a useful tool in the diagnostic process.

Recommendations for clinical use of WES

Since the introduction of WES, several novel variants in novel genes as well as known disease-causing genes have been identified in AD and FTD.[1, 36] The accomplishment of WES in the genetic research has also led to a rapid implementation of WES in the clinical setting due to its powerful and efficient approach to identify causative mutations. Nevertheless, the massive use of WES has also resulted in the identification of a large number of variants of unknown significance (VUS). Supporting evidence such as segregation studies or functional experiments for the pathogenicity of these variants is often absent. The question remains how to interpret these variants. Several studies have reported guidelines and standards aiming to assess the clinical relevance of genetic variants.[37-40] It is important to determine whether the gene function is affected by the variant of interest, and whether the phenotype of interest can be caused by functional change of the variant. Relevant information determining the relation of a variant to disease includes type of variants in a gene that are known to result in disease, disease phenotype(s) associated to gene, inheritance patterns of described variants for disease, protein domains implicated to pathogenesis of disease and described gene-phenotype correlations. Previous studies may provide us valuable information regarding functional change of protein affected by gene mutation and relation of

a gene to disease. Additionally, prevalence of disease, age at onset and inheritance mode of disease could help us further assessing the potential relevance of a variant in relation to disease. Variants with high allele frequencies in publically available data (i.e. Exome Aggregation Consortium, 1000 Genomes) were assumed to be less likely causing rare diseases.[41, 42] It is important to keep in mind that publically available data of genetic variation also includes presymptomatic individuals with late onset disease, asymptomatic individuals with low penetrance disease or individuals with younger than typical age at onset, and heterozygous carriers with recessive traits. At last, gene expression in various organs and underlying pathophysiology of the disease of interest may also help us to determine the potential involvement of a gene to disease.

Various in silico prediction programs have been developed to predict the pathogenicity of genetic variants.[40] Most prediction tools have been focused on missense variant including commonly used tools such as Polyphen-2,[43] SIFT,[44] MutationTaster.[45] Other prediction tools including CADD,[46] Genesplicer,[47] Human Splicing Finder,[48] could also predict non-coding variants and splice site variants. These prediction tools differ have distinct algorithms to predict possible effect of a variant, and each tool has their own strengths and weaknesses. Therefore, a combination of multiple tools should be considered to prioritize the candidate variants.

In general, genetic segregation provides a robust support for assessing the pathogenic effect of a variant, particularly when it segregates in extended families. However, DNA samples of additional family members are often limited or unavailable. In absence of genetic segregation, supporting functional experiments of a variant may provide evidence for the potential damaging effect of the variant. In vitro assays using cell models in particularly induced pluripotent stem cells, and animal models could provide insights in the disease mechanism.

Future perspective

Genetic research post-WES era

Although GWAS and WES have substantially been contributed to the identification of novel genetic mutations in AD and FTD,[49] a substantial part of the genetic contribution in these diseases remains unknown, with up to 57.7% of FTLD-TDP patients could not be explained by known mutations.[50] Due to various technical and analytic issues of WES (as described in our introduction), whole genome sequencing (WGS) has been suggested to be superior to WES in analyzing coding variants, copy number variants or structural variants. Several studies have successfully identified novel mutations in AD and FTLD using WGS.[50, 51] With decreasing costs, WGS has become more and more popular. Beyond mutations in coding regions, mutations in non-coding regions could be

involved in the pathophysiology of the disease.[52] Non-coding regions such as promotor, insulator and enhancers are essential for gene regulation. One could speculate that mutations in these regions could have a tremendous effect on gene expression. It can be hypothesized that at least some of the missing heritability may be explained by mutations in these non-coding regions.

In the genetic analysis of rare variants, large sample sizes in cases and controls are essential for increasing success rate to identify rare variants associated with disease. Various AD and FTD consortia have been set up to collect large number of cases and controls for rare variant analysis, and have successfully identified novel genetic association for AD and FTD.[50, 53, 54]

Sequencing analysis has usually been carried out on DNA extracted from blood, in particularly for clinical analysis of patients with neurodegenerative diseases, whose brain tissues were not available. However, mutations can be tissue-specific,[55] and the presence of genetic differences between cells among one single individual is possible, also referred as somatic mosaicism. The detection of these somatic mutations can be challenging due to unavailability of affected tissue. One example of somatic mosaicism is *C9orf72* repeat expansion in which higher repeat lengths have been observed in brain tissue than in blood.[56] However, normal *C9orf72* repeat lengths in blood but expanded repeats in brain tissue have not been reported so far. Somatic mosaic mutations in brain tissue containing a mixed of mutant cells and normal cells might result in aggregation of disease-specific protein locally, and as a subsequent lead to spreading of protein aggregates in a prion-like way.[55] This hypothesis is particularly of interest for sporadic disease without known mutations. Classical Sanger sequencing and arrays are often not sensitive enough to detect this low level of mosaic mutations. Recent development of NGS allows us to detect low mutation levels using ultra deep sequencing. One study has proved that ultra deep sequencing of brain region of AD patients could identify mosaic mutations in cells with an allele frequency as low as 1 percent.[57] Although the pathogenicity of these mosaic mutations is unknown, this study highlighted the presence of mosaic mutations in known AD mutations and indicated the possible involvement of these mutations in the development of disease.

The integration of NGS, mass spectometry technology and bioinformatic tools enabled us to study a disease traits at different levels encompassing genomics, epigenetic factors (epigenomics), RNA (transcriptomics), metabolites (metabolomics) and proteins (proteomics), and has allowed to investigate the relationship between distinct omics data.[58] The study of multi-omics data can help us to elucidate the pathomechanism of the disease, for example how alteration at genomic or epigenomic level might result in changes at metabolomic or proteomics level. This is of particularly interest for complex disease such as AD, in which multifactorial factors contribute to development of disease. We have demonstrated in **chapter 3.3** how combining WES (genomics) and proteomic

data could result in the discovery a novel mutation in neurodegeneration. Besides, it can also be a major step toward personalize medicine based on these multi-omics data.

Functional studies are still warranted to elucidate the exact disease mechanism. Promising methods such as induced pluripotent stem cells and CRISPR technology, studying a disease status by introducing the mutation of interest, can provide us insights in the disease mechanism how the mutation results in specific phenotype.[59]

Clinical implementation

In clinical setting, NGS has been applied to diseases with uncertain diagnosis, treatment and surveillance of devastating diseases such as cancer.[60] Due to clinical overlap of distinct neurodegenerative diseases, WES has increasingly been used for clinical diagnosis, even in sporadic cases. However, one should be cautious of classifying variants as damaging as this may have disastrous consequences for patients and families. Besides, unexpected incidental genetic findings for diseases unrelated to the observed phenotype, such as increasing risk for cancer, may result in dilemmas whether to report these findings to the patients or not. Nowadays, specific disease panels are often requested in clinical setting to avoid this problem. Clinicians should be aware of these problems before applying WES. Early communication about these issues to patients can avoid misunderstanding.

In cancer diagnostics, tumor tissue has been classified by their genetic changes rather than their originating tissue nowadays,[60] and this genetic profile of the tumor tissue is essential for determining the choice of treatment. Considering the clinical heterogeneity of AD and FTLD, genetic testing of patients is valuable to determine the correct diagnosis including the underlying pathomechanism, and therefore the choice of treatment. This is in particular of interest for FTLD, in which distinct genetic forms and molecular pathologies exist. With our increased understanding of gene mutations and its underlying disease mechanism, and genetic variations with modifying effect on risk of developing disease or disease course, it is conceivable that a pharmacogenomics profile may also be implemented in the therapy of AD and distinct FTLD forms in the future. Interestingly, a recent paper suggested that a single variant in phospholipase C gamma 2 (*PLCG2*) has a protective role in AD, FTLD and dementia with Lewy bodies, suggesting a shared etiology between these diseases and potential target for drug therapy.[61]

In conclusion, WES has made a major step in the genetic research of AD and FTD by identifying rare variants contributing to disease. Despite its advent in the genomics, a substantial part of the genetic contribution to AD and FTLD remained unexplained. Larger sample sizes for genetic associations studies, accompanying with the combina-

tion of different omics data will be essential for success in the expedition to identify remaining unknown genetic factors. Larger consortia of international research groups will be established aiming to collect reasonable sample sizes of cases and controls to increase the rate of success. In clinical setting, WES should increasingly be applied in diagnostic process, in particularly in patients with uncharacterized dementia and positive family history, and may replace standard Sanger sequencing in the future. Future therapeutic trials should consider determining genetic profiles using NGS data to stratify patients based on the presence of genetic factors, and to account for genetic modifiers as covariates to investigate the effect of drugs.

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CHAPTER 5

5

Summary
Samenvatting

Summary

Dementia is a disorder characterized by cognitive impairments and behavioral disturbances that interfere with ability of daily functioning, with Alzheimer's disease (AD) as the most common form of dementia, and frontotemporal dementia (FTD) as the second most common before age of 65. Both disorders are characterized by a clinical heterogeneity, in particularly for FTD patients, wherein symptoms suggestive for motor neuron disease (MND), corticobasal syndrome or progressive supranuclear palsy may occur. Genetic factors are involved in the development of AD and FTD. Approximately 5-10% of early-onset AD (EOAD) can be caused by autosomal dominant inherited mutations in presenilin 1 (*PSEN1*), presenilin 2 (*PSEN2*) and amyloid precursor protein (*APP*). FTD is a highly heritable disorder with familial forms in 30-50% of FTD patients. Mutations in microtubule associated protein tau (*MAPT*), progranulin (*GRN*) or hexanucleotide repeat expansion within the non-coding region of Chromosome 9 open reading frame 72 (*C9orf72*) can be found in up to 60% of familial FTD patients. Although a majority of familial forms could be linked to these highly penetrant mutations, a substantial part of familial cases still remained genetically unexplained.

In the last decade, advances in technology have enabled us to further investigate rare genetic variations using next-generation sequencing methods. Whole exome sequencing (WES), a next-generation sequencing technique, investigating rare variants (with minor allele frequency < 1%) in protein coding regions, is a time and cost-effectively method studying causative genetic mutations in patients with unknown genetic defects that are probably related to disease.

This thesis focuses on the finding of genetic causes of patients with AD and FTD with unknown genetic defects using whole exome sequencing, and describes the clinical and neuropathological features of these mutation carriers.

Chapter 1 provides the general introduction to the distinct genetic forms of AD and FTD with their clinical and pathological features and outlines the aims of this thesis.

Chapter 2 describes the study of underlying genetic factors using WES in patients with EOAD and/or familial AD. In **Chapter 2.1** we screened a Dutch cohort of 68 EOAD on rare variants in AD, FTD and prion disease genes, and found three *PSEN1* and two *PSEN2* variants. Of those, three variants were not reported previously, and were absent in control exomes. Although the clinical relevance of one variant was unclear, other variants were assumed to be probable pathogenic. Clinical presentation of the mutation carriers varied from typical memory complaints to behavioral changes or extrapyramidal signs. Our findings underlined the clinical heterogeneity among *PSEN1* and *PSEN2* carriers, which makes the clinical diagnosis challenging. In **Chapter 2.2** we examined eight AD families with suggestive autosomal dominant inheritance, unexplained by

mutations in *PSEN1*, *PSEN2* and *APP*. Using WES, we found two distinct rare variants in *EIF2AK3* gene, segregating with disease in two AD families, and nominated this gene as candidate gene for AD. The association of *EIF2AK3* with AD was further supported by an enrichment of rare variants in the WES data of Dutch individuals with AD compared to non-demented controls. Burden test by collapsing all rare variants in *EIF2AK3* showed almost two times increased risk of AD. This association was mainly driven by one variant p.Arg240His, which was also found in one of our AD family. Although we could not replicate the association of this variant with AD in an independent AD cohort, there was a trend towards association. *EIF2AK3* encodes for protein kinase RNA-like endoplasmic reticulum kinase (PERK), which functions as a sensor in the endoplasmic reticulum involved in the activation of the unfolded protein response (UPR) that is essential for protein homeostasis. PERK has been implicated to play a role in learning, memory and pathogenesis of AD. Neuropathological examination of *EIF2AK3* carriers showed an increased phosphorylated PERK and phosphorylated eukaryotic initiation factor-2α compared to non-demented controls, indicating an increased UPR as also found in previous studies of AD patients. However, the UPR activation was similar between *EIF2AK3* carriers and non-*EIF2AK3* carriers with AD. This is the first study supporting genetic association of rare *EIF2AK3* variants with AD. Future studies using larger sample sizes are needed to investigate this association. It is unclear how rare *EIF2AK3* variants contribute to AD. Functional studies are warranted to elucidate the exact disease mechanism.

Chapter 3 describes the genetic and pathological features of carriers of genetic variants in frontotemporal lobar degeneration (FTLD) identified by WES. In **Chapter 3.1** we reported two novel and one reported variants in valosin-containing protein (*VCP*) gene identified by WES and targeted sequencing of known causative genes involved in neurodegenerative disorders. These mutations were assumed to be pathogenic based on: 1) previous reported mutations in the same codons; 2) absence in healthy control exomes and public exome databases; 3) neuropathological findings of *VCP* carriers consistent with FTLD- transactive response DNA-binding protein (TDP) type D. Main clinical presentation of the carriers was behavioural changes, with additional semantic impairment in one patient, concomitant wit clinical diagnosis of behavioral FTD. No signs of amyotrophic lateral sclerosis, myopathy or Paget disease were noticed during the disease course making the classical *VCP*-mutation associated phenotype of inclusion body myositis with Paget's disease of the bone and frontotemporal dementia unlikely. Large clinical variability including variable age at onset and clinical presentation has been frequently found in *VCP* mutation carriers, even within the same family, and may indicate the presence of potential genetic modifiers involved in the disease course. In **Chapter 3.2** we provided the evidence of co-segregation of a novel heterozygous variant in Tubulin alpha 4A (*TUBA4A*) with disease in a family with FTD phenotype unexplained by mutations in known FTD genes. *TUBA4A* has been implicated to be a

rare genetic cause of ALS, but the supported evidence for genetic association with FTD was limited. In our FTD family, behavioral FTD without symptoms suggestive for ALS was the main clinical phenotype in most patients, except for one with uncharacterized dementia and extrapyramidal symptoms. Neuropathological findings corresponded with a mixed of FTLD-TDP type A and type B characteristics. Screening of *TUBA4A* in our FTD cohort did not identify additional variants. This is the first study describing co-segregation of a novel *TUBA4A* variant with disease in a FTD family with its underlying neuropathology. Our study indicates that *TUBA4A* could be a rare genetic cause of Dutch FTD patients.

Chapter 3.3 describes a large family with neurodegenerative disorder presenting with dementia and/or extrapyramidal symptoms. Combining genome-wide linkage analysis, WES and proteomic analysis we identified a novel variant (p.Leu50Arg) in protein kinase A type I-beta regulatory subunit (*PRKAR1B*) gene co-segregating with disease in the family. The variant is located in the dimerization/docking (D/D) domain of the regulatory subunits of the protein, and is predicted to hinder dimerization between the D/D domains within the protein kinase A (PKA) holoenzymes, as a result in an impaired function of PKA or in aggregation of PRKAR1B protein. Unique pathological findings were found in three carriers characterized by abundant neuronal cytoplasmatic inclusions positive stained for p62, neurofilament, α-internexin and PRKAR1B, but negative for fused in sarcoma (FUS), α-synuclein, and TDP43. Mutations in *PRKAR1B* are assumed to be rare as no pathogenic mutations in *PRKAR1B* has been identified in a cohort of patients with familial Parkinson's disease or FTD. In **Chapter 3.4** we screened an additional cohort of patients with clinical diagnosis of FTD (*n*=74) or AD (*n*=229) below the age of 70 on variants in *PRKAR1B* gene and known AD and FTD genes. We identified two rare nonsynonymous variants in *PRKAR1B*, probably not pathogenic due to benign prediction by in silico prediction tools. In our study, the observed mutation frequency in patients with AD was 5%, with *PSEN1* as most common. Higher mutation frequency of 14% was observed in FTD patients, with *C9orf72* mutation as most frequent. Mutations in FTD genes were found in patients with clinical diagnosis of AD.

In **Chapter 4** we discuss the main findings of this thesis, the clinical recommendations of WES and suggestions for future research.

Samenvatting

Dementie is een aandoening die gekenmerkt wordt door cognitieve stoornissen en gedragsveranderingen die interfereren met het dagelijks functioneren. De meest voorkomende vorm van demente is de ziekte van Alzheimer (ZvA). Frontotemporale dementie (FTD) is de tweede meest voorkomende vorm van dementie voor het 65e levensjaar. Beide vormen van dementie worden gekenmerkt door een heterogene klinische presentatie. Deze heterogene klinische presentatie is meest kenmerkend voor patiënten met FTD, waarin patiënten ook klachten van motorisch voorhoornlijden, corticobasaal syndroom of progressieve supranucleaire verlamming kunnen hebben. Genetische factoren zijn betrokken in de ontwikkeling van zowel ZvA als FTD. Ongeveer 5-10% van de preseniele ZvA wordt veroorzaakt door erfelijke mutaties in presenilin 1 (*PSEN1*), presenilin 2 (*PSEN2*) en amyloid precursor protein (*APP*) genen. FTD kan veroorzaakt worden door erfelijke mutaties in microtubule associated protein tau (*MAPT*), progranulin (*GRN*) and hexanucleotide repeat expansion in het niet-coderende deel van het chromosoom 9 open reading frame 72 (*C9orf72*) genen. In familiaire vorm van FTD, wordt tot 60% van de ziekte verklaard door mutaties in *MAPT*, *GRN* en *C9orf72* genen. Dit betekent dat er een deel van de ziekte nog geen erfelijke verklaring heeft.

In het laatste decennium hebben ontwikkelingen in technologie ons de mogelijkheid gegeven verder te verdiepen in het opsporen van zeldzame genetische varianten met behulp van next-generation sequencing technieken. Een voorbeeld hiervan is whole-exome sequencing (WES), waarmee men zeldzame genetische varianten (d.w.z. varianten met een allelfrequentie van kleiner dan 1% in eiwit coderende delen van het DNA kunnen opsporen. WES is een tijd- en kosteneffectieve methode om genetische mutaties op te sporen bij patiënten zonder bekende erfelijke defecten.

Dit proefschrift focust zich op het vinden van de genetische oorzaken van patiënten met ZvA en FTD zonder aangetoonde genetische mutatie met behulp van WES en het beschrijven van de klinische en neuropathologische kenmerken van deze genetische mutatiedragers.

Hoofdstuk 1 geeft een algemene introductie over de verschillende genetische vormen van ZvA en FTD met de bijhorende kliniek en neuropathologie en beschrijft de doelstelling van dit proefschrift.

Hoofstuk 2 beschrijft de studie naar de genetische factoren bij patiënten met preseniele ZvA en familiaire vorm van ZvA door middel van WES. In **hoofdstuk 2.1** hebben we een Nederlandse cohort van 68 patiënten met preseniele ZvA gescreend op bekende genen betroffen bij ZvA, FTD en prionziekte. Hierbij vonden we 3 genetische varianten in het *PSEN1* gen en 2 varianten in *PSEN2* gen. Drie van deze varianten zijn niet eerder beschreven en zijn afwezig in controles. Hoewel de klinische relevantie van 1 variant niet

duidelijk is, zijn de andere 2 varianten waarschijnlijk als pathogeen te beschouwen. De klinische presentatie van de mutatiedragers varieerde van klassieke geheugenstoornis tot gedragsproblemen of extrapiramidale symptomen. Onze bevindingen benadrukken de heterogene klinische presentatie voor patiënten met *PSEN1* en *PSEN2* mutaties. Dit kan het stellen van de juiste klinische diagnose bemoeilijken. In **hoofdstuk 2.2** hebben we 8 families met een waarschijnlijke autosomaal dominante vorm van de ZvA onderzocht waarvan de genetische oorzaak niet verklaard werd door mutaties in *PSEN1*, *PSEN2* en *APP* genen. Door middel van WES analyse hebben we 2 verschillende zeldzame varianten in het *EIF2AK3* gen gevonden. Deze varianten segregeerden met de ziekte in deze families. Zodoende hebben we dit gen genomineerd als mogelijke genetische oorzaak voor de ZvA. De genetische associatie van *EIF2AK3* gen is tevens ondersteund door de aanwezigheid van meerdere zeldzame varianten in het *EIF2AK3* gen in cohorten van patiënten met de ZvA in vergelijking met niet-demente individuen. De burden test, die alle zeldzame varianten in *EIF2AK3* bij elkaar neemt, toonde een bijna 2x verhoogde risico op het krijgen van de ZvA. Deze genetische associatie werd vooral veroorzaakt door de variant p.Arg240His, die ook in één van de families met ZvA werd gevonden. Hoewel we de genetische associatie van deze p.Arg240His variant niet konden bevestigen in een onafhankelijk cohort van patiënten met ZvA, was er wel een trend naar associatie. *EIF2AK3* codeert voor het eiwit protein kinase RNA-like endoplasmic reticulum kinase (PERK). Dit eiwit fungeert als een sensor in het endoplasmatisch reticulum, dat betrokken is bij de activatie van de unfolded protein response (UPR) welke belangrijk is voor de eiwithuishouding. Het is aangetoond dat PERK een rol speelt bij leren, geheugen en de pathogenese van de ZvA. Neuropathologisch onderzoek van de dragers met *EIF2AK3* varianten toonde een verhoogde fosforylatie van het PERK eiwit en het eukaryotic initiation factor-2α eiwit in vergelijking met niet-demente individuen. Dit wijst op een verhoogde UPR die eerder ook beschreven is bij studies van patiënten met de ZvA. Echter is deze verhoogde UPR vergelijkbaar in dragers en niet-dragers van de *EIF2AK3* varianten. Dit is de eerste studie die de genetische associatie van zeldzame varianten in het *EIF2AK3* gen met de ZvA ondersteunt. Toekomstige studies met grotere aantallen van patiënten en controles zijn nodig om deze associatie verder te onderzoeken. Het is vooralsnog niet geheel duidelijk hoe zeldzame *EIF2AK3* varianten betrokken zijn bij het ontstaan van de ZvA. Functionele studies zijn nodig om het exacte ziektemechanisme op te helderen.

Hoofdstuk 3 beschrijft de genetische en pathologische kenmerken van dragers van genetische varianten voor frontotemporale lobaire degeneratie (FTLD) die door middel van WES zijn gevonden. In **hoofdstuk 3.1** hebben we middels WES en gerichte sequencing (targeted sequencing) van bekende genetische oorzaken betrokken bij neurodegeneratieve ziekten een tweetal nieuwe en een bekende mutatie in het valosin-containing protein (*VCP*) gen gevonden. We beschouwen deze genetische mutaties als pathogeen op basis van 1) Eerdere mutaties die in het dezelfde eiwitcodon

zijn gerapporteerd; 2) afwezigheid van dezelfde mutaties in exomen data van gezonde controles en publiek toegankelijke exomen databases; 3) FTLD-transactive response DNA-binding (TDP) protein type D bij neuropathologisch onderzoek van de *VCP*-mutatiedragers. De klinische presentatie van gedragsverandering, met tevens bijkomende semantische taalstoornissen bij een patiënt, paste bij de klinische diagnose van gedragsvariant FTD. Klinische tekenen van amyotrofische laterale sclerose (ALS), myopathie of de ziekte van Paget werden niet gevonden in het ziektebeloop. Dit maakte de klassieke *VCP* genmutatie geassocieerde klinische diagnose van inclusion body myositis met de ziekte van Paget en frontotemporale dementie minder waarschijnlijk. *VCP* gen mutatiedragers worden gekenmerkt door grote heterogene klinische presentatie inclusief de variabele debuutleeftijd en verschillende klinische symptomen, zelfs bij individuen binnen dezelfde familie. Dit suggereert de aanwezigheid van potentiële genetische factoren die het ziekteverloop kunnen beïnvloeden.

In **hoofdstuk 3.2** tonen we bewijs voor de segregatie van een nieuwe variant in het Tubuline alpha 4A (*TUBA4A*) gen in een FTD familie zonder bekende genetische oorzaak. *TUBA4A* is beschreven als een zeldzame genetische oorzaak voor ALS, maar het bewijs voor de betrokkenheid van dit gen bij FTD is gering. Gedragsvariant FTD zonder klinisch symptomen van ALS was het meest voorkomende fenotype in deze familie. Eén patiënt had een niet nader gespecificeerde dementie met extrapiramidale verschijnselen. Bevindingen bij pathologisch onderzoek pasten bij een mix van FTLD-TDP type A en type B. De screening van *TUBA4A* gen in onze FTD cohort toonde geen overige potentiële pathogene varianten. Dit is de eerste studie die de segregatie van een nieuwe *TUBA4A* gen variant met de bijbehorende pathologie beschrijft. Onze studie suggereert dat mutaties in het *TUBA4A* gen mogelijk een zeldzame genetische oorzaak is voor FTD.

Hoofdstuk 3.3 beschrijft een grote familie met neurodegeneratieve ziekte waarbij de patiënten zich presenteren met dementie en/of extrapiramidale verschijnselen. Door een gecombineerde analyse van familie koppelingsonderzoek, WES en proteomic onderzoek hebben we een nieuwe variant (p.Leu50Arg) in het protein kinase A type I-beta regulatory subunit (*PRKAR1B*) gen gevonden wat segregeerde met de ziekte in deze familie. De variant bevindt zich in het dimerisatie/docking domein van de regulatoire subunit van het eiwit. In silico analyse voorspelt dat deze variant de verbinding van de dimerisatie/docking domein binnen de proteine kinase A (PKA) holoenzym kan verstoren. Dit kan resulteren in een gestoorde PKA functie of in ophopingen van PRKAR1B eiwitten. De unieke neuropathologische bevindingen, gekenmerkt door overvloed aan neuronale cytoplasmatische eiwitophopingen die een positieve kleuring hebben voor p62, neurofilament en α-internexin eiwitten, maar negatief zijn voor fused in sarcoma (FUS), α-synucleine, and TDP43 eiwitten. De veronderstelling is dat *PRKAR1B* gen mutatie zeldzaam is in FTD en de ziekte van

Parkinson omdat er geen overige pathogene mutaties zijn gevonden in een cohort met FTD en Parkinson patiënten. In **hoofdstuk 3.4** hebben we een additioneel cohort met patiënten met klinische diagnose van FTD (*n*=74) of ZvA (*n*=229) (onder 70 jarige leeftijd) getest op varianten in het *PRKAR1B* gen. Hierbij vonden we tweetal zeldzame nonsynoymous varianten in *PRKAR1B* gen die waarschijnlijk niet pathogeen zijn. We vonden een mutatiefrequentie van 5% in patiënten met de ZvA en *PSEN1* was de meest voorkomende mutatie. De mutatiefrequentie was hoger in FTD patiënten met *C9orf72* repeat expansie als de meest voorkomende mutatie. Mutaties in FTD genen werden gevonden in patiënten met klinische diagnose van ZvA.

In **hoofdstuk 4** bespreken wij de belangrijkste bevindingen in dit proefschrift, de aanbevelingen voor de klinische toepassing van WES en het toekomstperspectief voor genetische onderzoek.

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About the author

Tsz Hang Wong was born on July 27th, 1985 in Hong Kong, China. After finishing secondary school at Segbroek College in The Hague in 2005, he started medical school at Erasmus University Rotterdam. During his study he participated in a research project on progressive supranuclear palsy under supervision of Prof. dr. J.C. van Swieten and dr. W.Z. Chiu at the department of Neurology at Erasmus Medical Center Rotterdam. After obtaining his medical degree in 2012, he started working on the PhD project Genetics of Alzheimer's disease and frontotemporal lobar degeneration, as presented in this thesis, at the department of Neurology at Erasmus Medical Center Rotterdam. During his PhD period he also worked in the laboratory at the Department of Neuroscience of Mayo Clinic (Jacksonville, USA), supervised by Rosa Rademakers.

After his PhD period, he has worked as medical doctor in Neurology at Erasmus Medical Center (Rotterdam) and Hagaziekenhuis (The Hague). In 2020 he started his residency in Neurology at Medisch Spectrum Twente (Enschede). Currently, he lives in Enschede with his wife and two daughters.

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PhD portfolio – Summary of PhD training and teaching activities

	Year	Workload (ECTS)
1. PhD training		
Research skills		
Erasmus Summer Programme	2012	3.5
Basiscursus klinische onderzoekers (BROK)	2013	1.4
Biostatistical Methods I	2014	1.4
Biomedical English writing and communication	2014	3
R course, MOLMED, Erasmus MC	2015	1.4
In-depth courses		
Advances in Genome Wide Association Studies, NIHES, Erasmus MC	2012	1.4
Family-based genetic analysis, Erasmus MC	2012	1.4
The SNP Course IX, MOLMED, Erasmus MC	2012	1.4
A first encounter with next-generation sequencing data, NIHES, Erasmus MC	2012	1.4
CME Course Neurodegenerative Diseases, Academic Medical Center, Amsterdam	2014	1.4
Exome sequencing, Wellcome Trust Sanger Institute, Hinxton, Engeland	2014	2.5
Design and analysis of genetic-based association studies, Wellcome Trust Sanger Institute, Hinxton, Engeland	2015	1.4
International conferences		
The 8th Conference on Frontotemporal Dementias, Manchester; Poster presentation	2012	1
Alzheimer's Association International Conference, Boston; Poster presentation	2013	1
American society of Human Genetics, San Diego; Poster presentation	2014	1
The 9th International Conference on Frontotemporal Dementias, Vancouver; Poster presentation	2014	1
Alzheimer's Association International Conference, Washington DC; Poster presentation	2015	1
Seminars workshops and other		
FTD expert groep meetings	2014	0.5
Alzheimer's disease Exome Sequencing Workgroup & Meetings	2013-2015	2
RIMOD FTD Workgroup & Meetings	2014-2015	1.5
Center for Individualized Medicine grant, Mayo Clinic: $ 50.000	2015	1
Fellowshipsbeurs buitenland, Alzheimer Nederland: € 25.000	2015	1
2. Teaching		
Lecturing		
Alzheimer Nederland Mix & Match, 'WES in neurofilamentopathie'	2013	0.3
'WES in neurodegenerative disease', research master programme	2014	0.3
Supervising		
Master student	2013	1.0
Master student	2014	2.0
Total		**36.2**

List of abbreviations

ABCA7	ATP Binding Cassette Subfamily A Member 7
AD	Alzheimer's disease
AKAP	A-kinase anchoring protein
ALS	Amyotrophic lateral sclerosis
APOE	Apolipoprotein E
APP	Amyloid precursor protein
ATF6	Activating Transcription Factor 6
Aβ40	Amyloid-beta 40
Aβ42	Amyloid-beta 42
BACE1	β-site APP cleaving enzyme 1
BIBD	Basophilic inclusion body disease
BvFTD	Behavioral variant FTD
C9orf72	Chromosome 9 open reading frame 72
CA	Cornu ammonis
CAA	Cerebral amyloid angiopathy
CADD	Combined annotation-dependent depletion
CBS	Corticobasal syndrome
CERAD	Consortium to Establish a Registry for AD
CHAPS	Cholamidopropyl dimethylammonio-1-propanesulphonate
CHCHD10	Coiled-coil-helix-coiled-coil-helix domain-containing protein 10
CHMP2B	Charged multivesicular body protein 2B
CSF	Cerebrospinal fluid
CT	Computed tomography
CTSC/RAB38	Cathepsin C/Ras-related protein Rab-38
D/D	Dimerization/docking
DLB	Dementia with Lewy bodies
DN	Dystrophic neurites
DPR	Dipeptide repeat proteins
EIF2AK3	Eukaryotic Translation Initiation Factor 2 Alpha Kinase 3
EOAD	Early-onset AD
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated protein degradation
ESP	Exome Sequencing Project
EVS	Exome Variant Server
EWS	Ewing Sarcoma
ExAC	Exome Aggregation Consortium
FET	Fused in Sarcoma, Ewing Sarcoma and TATA binding associated factor 15

FTD	Frontotemporal dementia
FTLD	Frontotemporal lobar degeneration
FUS	Fused in sarcoma
G4C2	GGGGCC
GATK	Genome Analysis Tool Kit
GBA	Glucocerebrosidase
GGT	Globular glial tauopathy
gnomAD	Genome aggregation database
GoNL	Genome of the Netherlands
GRN	Progranulin
GSK3β	Glycogen synthase kinase 3β
GWAS	Genome-wide association studies
HEX	Healthy EXomes
hnRNPA1	Heterogeneous nuclear ribonucleoproteins A1
hnRNPA2B1	Heterogeneous nuclear ribonucleoproteins A2B1
HSP	Hereditary Spastic Paraplegia
IBMPFD	Inclusion body myopathy with Paget's disease of the bone and frontotemporal dementia
IF	Intermediate filaments
IR	Immunoreactive
IRE1	Inositol Regulating Enzyme 1
LB	Lewy bodies
LGMD	Limb-Girdle Muscular Dystrophy
LOAD	Late-onset AD
LoF	Loss of function
LS	Low salt
MAF	Minor allele frequency
MAPT	Microtubule associated protein tau
MLPA	Multiplex ligation-dependent probe amplification
MMSE	Mini Mental State examination
MND	Motor neuron disease
MRI	Molecular mass
MSP	Multisystem proteinopathy
NCI	Neuronal cytoplasmic inclusions
NF	Neurofilament
NGS	Next-generation sequencing
NIFID	Neuronal intermediate filament inclusion disease
NII	Neuronal intranuclear inclusions
NOS	Not otherwise specified
OPTN	Optineurin

PD	Parkinson's disease
PDB	Paget disease of the bone
peIF2α	Phosphorylated eukaryotic initiation factor-2α
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
PGRN	Progranulin (protein)
PKA	Protein kinase A
PLCG2	Phospholipase Cγ2
PLD3	Phospholipase D3
PNFA	Progressive non-fluent aphasia
PPA	Primary progressive aphasia
pPERK	Phosphorylated pancreatic endoplasmic reticulum kinase
PRKAR1B	Protein kinase A type I-beta regulatory subunit
PSEN1	Presenilin 1
PSEN2	Presenilin 2
PSP	Progressive supranuclear palsy
PTV	Protein truncating variants
RAN	Repeat- associated non-ATG
RS	Rotterdam study
SA	Sarkosyl
SD	Semantic dementia
SIFT	Sorting Intolerant from Tolerant
SNP	Single nucleotide polymorphism
SORL1	Sortilin-related receptor 1
SPECT	Single photo emission computed tomography
SQSTM1	Sequestome 1
svFTD	Semantic variant frontotemporal dementia
TAF15	TATA-binding protein-associated factor 15
TARDBP	TAR DNA binding protein
TBK1	TANK-binding kinase 1
TDP	Transactive response DNA-binding protein
TDP-43	Transactive response DNA-binding protein of 43 kDa
TMEM106B	Transmembrane protein 106B
TREM2	Triggering Receptor Expressed on Myeloid Cells 2
TUBA4A	Tubulin alpha 4A
TX	TritonTM X-100
UBQLN2	Ubiquilin 2
UNC5C	Unc-5 netrin receptor C
UPR	Unfolded protein response
UPS	Ubiquitin proteasome system
UR	Urea

VCP	Valosin-containing protein
VQSR	Variant Quality Score Recalibration
VUS	Variant with uncertain significance
WES	Whole exome sequencing
WGS	Whole genome sequencing

Whole exome sequencing in Alzheimer's disease and frontotemporal lobar degeneration

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