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Original Article
C9ORF72 repeat expansions and other FTD gene mutations in a clinical AD patient series from Mayo Clinic

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Abstract: Alzheimer disease (AD) and frontotemporal dementia (FTD) are two frequent forms of primary neurodegenerative dementias with overlapping clinical symptoms. Pathogenic mutations of the amyloid precursor protein (*APP*) and presenilins 1 and 2 (*PSEN1*, *PSEN2*) genes have been linked to familial early-onset forms of AD; however, more recently mutations in the common FTD genes encoding the microtubule associated protein tau (*MAPT*), progranulin (*GRN*) and *C9ORF72*, have also been reported in clinically diagnosed AD patients. To access the contribution of mutations in a well-characterized series of patients, we systematically performed genetic analyses of these EOAD and FTD genes in a novel cohort of 227 unrelated probands clinically diagnosed as probable AD which were ascertained at Mayo Clinic Florida between 1997 and 2011. All patients showed first symptoms of dementia before 70 years. We identified 9 different pathogenic mutations in the EOAD genes in a total of 11 patients explaining 4.8% of the patient population. Two mutations were novel: PSEN1 p.Pro218Leu and PSEN2 p.Phe183Ser. Importantly, mutations were also identified in all FTD genes: one patient carried a MAPT p.R406W mutation, one patient carried the p.Arg198Glyfs19X loss-of-function mutation in GRN and two patients were found to carry expanded GGGGCC repeats in the non-coding region of *C9ORF72*. Together the FTD genes explained the disease in 1.8% of our probable AD population. The identification of mutations in all major FTD genes in this novel cohort of clinically diagnosed AD patients underlines the challenges associated with the differential diagnosis of AD and FTD resulting from overlapping symptomatology and has important implications for molecular diagnostic testing and genetic counseling of clinically diagnosed AD patients. Our findings suggest that in clinically diagnosed AD patients, genetic analyses should include not only the well-established EOAD genes *APP*, *PSEN1* and *PSEN2* but also genes that are usually associated with FTD. Finally, the overall low frequency of mutation carriers observed in our study (6.6%) suggests the involvement of other as yet unknown genetic factors associated with AD.

Keywords: Alzheimer's disease, frontotemporal dementia, amyloid precursor protein, presenilin 1, presenilin 2, progranulin, microtubule associated protein tau, C9ORF72, mutation, diagnosis.

Introduction

Alzheimer's disease (AD) is the most common type of neurodegenerative dementia, clinically characterized by progressive memory impairment and deficits in cognitive functions [1]. A definite diagnosis of AD can only be obtained at autopsy. In addition to overt neuronal loss, AD is pathologically characterized by extracellular deposition of β-amyloid (Aβ) in senile plaques and the formation of intracellular neurofibrillary tangles mainly composed of hyperphosphorylated microtubule associated protein tau. The distinction between the rare early-onset (presenile, EOAD) and common late-onset (senile, LOAD) form of AD is based on the age when first clinical symptoms appear, and usually 65 years is used as an arbitrary cut-off age. EOAD patients are clinically and pathologically identical to LOAD patients, although the disease progression may be more rapid and the brain pathology more pronounced in EOAD patients.

AD is a genetically complex disorder. In the last two decades, pathogenic mutations leading to autosomal dominant EOAD have been identified in the Aβ precursor protein (*APP*) gene and the presenilins 1 and 2 (*PSEN1*, *PSEN2*) genes [2-

5]. Studies of cellular and mouse models have shown that mutations in these genes cause an increased production of the neurotoxic $A\beta_{42}$ [6], indicating that unbalanced APP processing might be the primary event leading to neurodegenerative brain pathology in AD patients carrying these mutations. In addition to these causal genes, the ε4 allele of the apolipoprotein E (*APOE*) gene has been established as a major risk factor for both familial and sporadic EOAD and LOAD patients [7-9]. More recently, genetic studies of AD have focused on the identification of common variants associated with risk for LOAD through genome-wide association studies. These studies have identified several new genes that show significant association with LOAD, including *CLU*, *BIN1*, *PICALM* and *ABCA7* among others, albeit each with only a small effect size [10-14].

The clinical symptoms of AD display a substantial overlap with symptoms observed in frontotemporal dementia (FTD), the second most common form of presenile dementia after AD [15, 16]. FTD is clinically associated with behavior/personality changes and language disturbances [17, 18]. The most common neuropathology associated with FTD is frontotemporal lobar degeneration (FTLD) with ubiquitin and TAR DNA-binding protein 43 (TDP-43) positive inclusions (FTLD-TDP) [19]. In recent years, significant progress has been made in the identification of causal FTD genes and mutations in the microtubule associated protein tau (*MAPT*), progranulin (*GRN*) and chromosome 9 open reading frame 72 (*C9ORF72*), now explain at least 20-30% of the familial and 5-10% of all sporadic FTD patients [20-26].

Due to the heterogeneity and wide range of clinical presentation of both AD and FTD, these disorders can share common features [27], particularly in patients with an early disease onset. Therefore, despite our increased understanding of clinical and pathological aspects of AD and FTD, the distinction between these presenile dementias is still difficult and may lead to a clinical pathological mismatch, that is, patients presenting with an amnestic syndrome may have TDP-43 or tau pathology and those presenting with behavior symptoms may have Alzheimer pathology, predominantly in the frontal lobes.

In this study, we genetically characterize a newly ascertained cohort of 227 clinical probable AD patients seen at Mayo Clinic, all with first symptoms of dementia before 70 years of age, for mutations in the common AD and FTD genes. We report mutations in all known AD and FTD genes, providing important implications for molecular diagnostic testing and genetic counseling of clinically diagnosed AD patients.

Materials and methods

Subjects

Our study cohort consisted of 227 unrelated EOAD patients (40.1% males and 59.9% females) from a clinical case series diagnosed at the Department of Neurology at Mayo Clinic Florida between 1997 and 2011. The majority of individuals tested in this study were Caucasian (90%), 21 individuals (9%) were African American and 2 (1%) were Asian. For all patients the diagnosis of probable AD was established according to the NINCDS-ADRDA criteria [28]. Autopsy was performed in 9 patients, confirming the diagnosis of AD in all. The onset of dementia was before 70 years of age in all patients, with a mean age at onset of 59.8 ± 7.0 years (range 34-69 years). In 142 patients (63% of the cohort), the onset of dementia was before the age of 65. A positive family history defined as having at least one affected first- or second degree relative with dementia was found in 128 patients (56%). For genetic studies, a total of 641 healthy controls collected at the Department of Neurology at Mayo Clinic Florida were also available. All subjects agreed to participate in the study and biological samples were obtained after written informed consent.

Sequencing analysis

Genomic DNA was isolated from blood samples using standard procedures. For each patient, PCR was performed for exon 16 and 17 of *APP*, exons 3-12 of *PSEN1* and exons 3-12 of *PSEN2* (primer sequences available upon request). PCR reactions were performed using 50 ng genomic DNA, 10x PCR Buffer (Quiagen) containing 15mM $MgCl_2$, 5mM dNTPs, 10 mM of each primer and 1U Taq polymerase (Apex). Each PCR product was purified using the AMPure system (Agencourt Bioscience Corporation, Beverly, MA, USA) and sequenced using the same forward and reverse primers with Big Dye terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). Sequencing products were purified using the CleanSEQ system

Table 1. TaqMan genotyping assays used to genotype *APOE*, *MAPT*, and *PSEN1*

Gene (position)	dbSNP ID	ABI TaqMan assay	Direction	PCR primers	Reporter primers (dye)
APOE 526T>C (p.Cys176Arg)	rs7412	C_904973_10	N/A	N/A	N/A
APOE 388C>T (p.Arg130Cys)	rs429358	C_3084793_20	N/A	N/A	N/A
MAPT 1216C>T (p.Arg406Trp)	rs63750424	C_27537056_10	N/A	N/A	N/A
PSEN1 653C>T (p.Pro218Leu)	NA	Custom assay	Forward Reverse	GAATTTTGGTGTGGTGGGAATGATT GCCATGAGGGCACTAATCATAATGA	AGTCGAAGTGGACCTTT (VIC) AGTCGAAGTAGACCTTT (FAM)

APOE, apolipoprotein E, *PSEN1*, presenilin 1, *PSEN2*, presenilin 2, N/A, not applicable.

(Agencourt Bioscience Corporation) and run on an ABI 3730 Genetic Analyzer (Applied Biosystems). The sequences were analyzed using Sequencher software (Gene Codes, Ann Arbor, MI, USA). Sequencing analyses of *PSEN2* exon 6 was also performed in all control individuals to exclude the presence of the novel c.548T>C (p.Phe183Ser) in our control population.

Genotyping analysis

APOE genotypes were determined with pre-designed TaqMan SNP genotyping assays for rs7412 and rs429358 (Applied Biosystems, Foster City, CA, USA) and analyzed on an ABI 7900HT Fast Real Time PCR system using Sequence Detection System 2.2.2 software (Applied Biosystems). The presence of the c.1216C>T (p.Arg406Trp) mutation in the *MAPT* gene was determined using a custom designed TaqMan SNP genotyping assay and confirmed for one heterozygous individual using PCR and sequencing analysis of *MAPT* exon 13. A custom designed TaqMan SNP genotyping assays was also used to study the presence of the novel mutation c.653C>T (p.Pro218Leu) in exon 7 of *PSEN1* in control individuals. Details on all custom and pre-designed Taqman assays are summarized in Table 1.

APP copy-number analysis

To detect genomic *APP* copy-number mutations, real-time PCR analysis was performed with a made to order TaqMan assay (Hs01547105_cn, Applied Biosystems) and analyzed on ABI7900HT Fast Real Time PCR system using SDS 2.2.2 software (ΔΔct method). Following manufacturer's protocol, 20ng genomic DNA from each patient was run in duplicate and normalized to the Copy Number Reference Assay RNase P (cat: 4403326, Applied Biosystems). Two previously identified patients, each carrying an APP duplication were included as a positive controls.

Screening for GGGGCC repeat expansions in C9ORF72

The GGGGCC hexanucleotide repeat expansion in *C9ORF72* was detected using a two-step PCR based protocol, as previously described [22]. Briefly, the hexanucleotide repeat was amplified in all samples using one fluorescently labeled PCR primer. Next, fragment length analysis was performed on an automated ABI3730 DNA analyzer using GeneMapper software (Applied Biosystems). All patients that appeared homozygous in this assay were next analyzed using a repeat primed PCR method where characteristic stutter amplification pattern on electropherogram was considered evidence of a pathogenic *C9ORF72* expansion.

GRN expression analyses

Plasma samples were available for 196 patients (86% of the cohort). GRN levels in 72 of these patients were analyzed and described previously [29]. For the remaining samples GRN expression levels were determined using the human Progranulin ELISA kit (Adipogen Inc., Seoul, Korea) using a 1:100 dilution of the plasma samples in 1x diluent following manufacturer's instructions. To increase accuracy all samples were analyzed twice in two independent experiments. Recombinant human GRN provided with the ELISA kit was used as a standard.

Results

Mutation analyses of EOAD genes

In the 227 clinical AD patients we identified 9 different pathogenic missense mutations in *APP, PSEN1* and *PSEN2* genes in a total of 11

Table 2. Pathogenic mutations identified in EOAD and FTD genes in this study

Patient ID	Gene	Exon	Genomic mutation	Predicted cDNA	Predicted protein	Protein domain	*APOE* genotype
506	*APP*	17	g.275333C>T	c.2141C>T	p.Thr714Ile	TM-I	ε3/ε3
11323[a]	*PSEN1*	5	g.25607A>G	c.404A>G	p.Asn135Ser	TM-II	-
9018	*PSEN1*	5	g.25639A>C	c.436A>C	p.Met146Leu	TM-II	ε2/ε3
711	*PSEN1*	7	g.44636G>C	c.617G>C	p.Gly206Ala	TM-IV	ε4/ε4
15066	*PSEN1*	7	g.44636G>C	c.617G>C	p.Gly206Ala	TM-IV	ε2/ε3
11967	*PSEN1*	7	g.44636G>C	c.617G>C	p.Gly206Ala	TM-IV	ε3/ε3
10232	*PSEN1*	7	g.44672C>T	c.653C>T	p.Pro218Leu[c]	TL-IV	ε3/ε3
15408	*PSEN1*	7	g.44733C>G	c.714C>G	p.Ile238Met	TM-V	ε3/ε4
14915	*PSEN2*	5	g.3661C>T	c.389C>T	p.Ser130Leu	HL-I	ε3/ε3
8120	*PSEN2*	6	g.6206A>G	c.520A>G	p.Met174Val	TM-III	ε4/ε4
15229	*PSEN2*	6	g.6234T>C	c.548T>C	p.Phe183Ser[c]	C-Term	ε3/ε3
14330[b]	*GRN*	6	g.1407_1408delAG	c.592_593delAG	p.Arg198Glyfs19X	InterFB	ε3/ε4
15625	*MAPT*	13	g.137465C>T	c.1216C>T	p.Arg406Trp	C-Term	ε3/ε3
9979	*C9ORF72*	Intronic	g.26724GGGGCC(3_23)	N/A	N/A	Non-coding	ε3/ε3
7391	*C9ORF72*	Intronic	g.26724GGGGCC(3_23)	N/A	N/A	Non-coding	ε3/ε3

[a] Patient previously reported in Crook et al. (ref). [b] Patient previously reported in Finch et al. (ref). [c] Novel mutation.
APP, amyloid precursor protein, *PSEN1*, presenilin 1, *PSEN2*, presenilin 2, *GRN*, progranulin, *MAPT*, microtubule associated protein tau, *C9ORF72*, chromosome 9 open reading frame 72. N/A, not applicable, TM, transmembrane domain, HL, hydrophilic loop, C-Term, C-terminal domain, InterFB, linker region between granulins F and B.

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                          160                              183                    200
Human PSEN2 WT            YKYRCYKFIHGWLIMSSLMLLFLFTYIYLGEVLKTYNVAMD
Human PSEN2 p.F183S       YKYRCYKFIHGWLIMSSLMLLFLSTYIYLGEVLKTYNVAMD
Chimpanzee PSEN2          YKYRCYKFIHGWLIMSSLMLLFLFTYIYLGEVLKTYNVAMD
Cow PSEN2                 YKYRCYKFIHGWLIMSSLMLLFLFTYIYLGEVLKTYNVAMD
Mouse PSEN2               YKYRCYKFIHGWLIMSSLMLLFLFTYIYLGEVLKTYNVAMD
Rat PSEN2                 YKYRCYKFIHGWLIMSSLMLLFLFTYIYLGEVFKTYNVAMD
Chicken PSEN2             YKYRCYKFIHGWLILSSFMLLFLFTYIYLGEVLKTYNVAMD
H. sapiens PSEN1          YKYRCYKVIHAWLIISSLLLLFFFSFIYLGEVFKTYNVAVD
p.F177S previously reported as pathogenic mutation in PSEN1
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Figure 1. Evolutionary sequence conservation of novel *PSEN2* mutation p.Phe183Ser and homology with *PSEN1*. ClustalW alignment for the part of *PSEN2* with the novel p.F183S mutation. Shown are human PSEN2 (NP_036618.2), chimpanzee PSEN2 (XP_001142009.2), cow PSEN2 (NP_776865.2), mouse PSEN2 (NP_001122077.1), rat PSEN2 (NP_112349.2), chicken (NP_989633.1) and human PSEN1 (NP_000012.1). Amino acid positions of PSEN2 codons are indicated above the alignment. Position 183 in PSEN2 corresponds with position 177 in PSEN1 previously reported to carry the corresponding p.Phe177Ser pathogenic mutation (ref).

patients (**Table 2**). In *APP*, we observed the known p.Thr714Ile mutation in exon 17, previously reported in an Austrian family [30], in one patient. No duplications of the *APP* gene were found. In *PSEN1*, we identified 4 known missense mutations (p.Asn135Ser, p.Met146Leu, p.Gly206Ala, p.Ile238Met) and 1 novel mutation (p.Pro218Leu), in a total of 7 affected individuals. All mutations were identified in a single patient except p.Gly206Ala which was detected in 3 unrelated patients. In *PSEN2* we found 3 missense mutations, two known (p.Ser130Leu, p.Met174Val) and one novel (p.Phe183Ser) in one patient each. The presence of the two novel mutations (*PSEN1* p.Pro218Leu and *PSEN2* p.Phe183Ser) was subsequently excluded in 641 healthy control individuals ascertained at Mayo Clinic. Both novel mutations are located in highly conserved residues of the PSEN1 and PSEN2 proteins, respectively (**Figure 1**). Moreover, protein sequence alignment of the two presenilins showed that amino acid residue Phe183 in PSEN2 corresponds to Phe177 in PSEN1. This codon in PSEN1 was previously reported to carry a pathogenic p.Phe177Ser mutation [31], further supporting the pathogenic character of this novel PSEN2 mutation (**Figure 1**). In addition to the pathogenic missense mutations, we identified four coding variants in exon 4 of *PSEN2,* all encoding amino acid changes in the poorly conserved N-terminal domain: 1 known variant of unknown significance (p.Arg62His) in 4 patients and 3 novel variants (p.Glu54Asp, p.Arg62Gly and p.Gly70Arg) in one patient each (**Table 3**). We

Table 3. Coding sequence variants in EOAD genes with unknown significance

Gene	Exon	Genomic mutation	Predicted cDNA	Predicted protein	Protein domain	rs number	Frequency in patients (%)
PSEN1	9	g.58389A>G	c.953A>G	p.Glu318Gly	HL-VI b	rs17125721	8 (3.5)
PSEN2	4	g.1816G>C	c.162G>C	p.Glu54Asp[a]	N-Term	-	1 (0.4)
PSEN2	4	g.1838C>G	c.184C>G	p.Arg62Gly[a]	N-Term	-	1 (0.4)
PSEN2	4	g.1839G>A	c.185G>A	p.Arg62His	N-Term	rs58973334	4 (1.7)
PSEN2	4	g.1862G>A	c.208G>A	p.Gly70Arg[a]	N-Term	-	1 (0.4)

[a] Novel mutation. *PSEN1*, presenilin 1, *PSEN2*, presenilin 2.

Table 4. *APOE* genotyping results in clinical AD patient series.

Age (years)	*APOE* genotype					
	ε2/ ε2 N (%)	ε2/ε3 N (%)	ε2/ε4 N (%)	ε3/ε3 N (%)	ε3/ε4 N (%)	ε4/ε4 N (%)
≤50	-	2 (7.5)	-	12(44.4)	12(44.4)	1(3.7)
51-55	-	2(5.5)	-	13(35.1)	17(45.9)	5(13.5)
56-60	-	2(5.0)	-	16(40.0)	15(37.5)	7(17.5)
61-65	-	2(3.1)	1(1.6)	19(29.7)	26(40.6)	16(25.0)
66-69	-	2(3.6)	1(1.8)	11(19.6)	23(41.1)	19(33.9)
Total	0	10(4.5)	2(0.9)	71(31.7)	93(41.5)	48(21.4)

also observed the common non-pathogenic polymorphism, PSEN1 p.Glu318Gly, in 8 patients (3.5% of the population).

APOE genotyping

In our series, 95 (42.4%) patients carried one *APOE* ε4 allele and 48 (21.4%) patients were homozygote for *APOE* ε4 (**Table 4**). The frequency of ε4/ε4 homozygotes increased with increasing age at onset. Although we did not perform a classical case-control association study, these frequencies are comparable to other AD patient series and confirm that *APOE* ε4 is a major risk factor in our series [9, 32].

Mutation analyses of FTD genes

To determine the contribution of FTD gene mutations to disease in our clinical AD cohort, we further performed mutation analyses of the common FTD genes *GRN*, *MAPT* and *C9ORF72* and identified 4 clinical probable AD patients carrying pathogenic mutations in FTD genes. The presence of pathogenic *GRN* mutations or deletions was determined using a GRN ELISA in plasma samples in 196 patients. Reduced levels of GRN (74 ng/ml) were observed in one patient, whereas GRN levels in the other AD patients were within the normal range. Sequencing analyses had previously confirmed the presence of a pathogenic loss-of-function mutation c.592_593delAG (p.Arg198Glyfs19X) in this patient [29]. In *MAPT*, we identified one patient carrying the c.1216C>T (p.Arg406Trp) missense mutation in exon 13. Finally, we analyzed our cohort for the presence of repeat expansions in *C9ORF72*. Out of the 227 patients, 91 were homozygous in our initial fluorescent PCR assay and were therefore analyzed using our *C9ORF72* repeat-primed PCR assay. Using this assay, two patients were found to carry the characteristic stutter pattern indicative of a pathogenic repeat expansion (**Figure 2**). Based on the repeat-primed PCR assay, patient 7391 is likely to carry a relatively small repeat expansion of about 60 repeats in this particular DNA sample extracted from blood cells, whereas the stutter pattern of patient 9979 is comparable to all previously published patients, known to carry 700-1600 copies of the GGGGCC repeat.

Clinical characteristics of mutation carriers

The clinical characteristics of the 15 clinical probable AD patients with pathogenic mutations identified in this study are summarized in **Table 5**. There was significant difference in age at onset between patients with *APP* and *PSEN* mutations (mean age 49.4 ± 8.1 years) and patients with mutations in FTD genes (mean age 62.3 ± 8.7 years). Among all mutations carriers, 10 patients reported at least one first-degree relative with dementia, 4 patients were sporadic

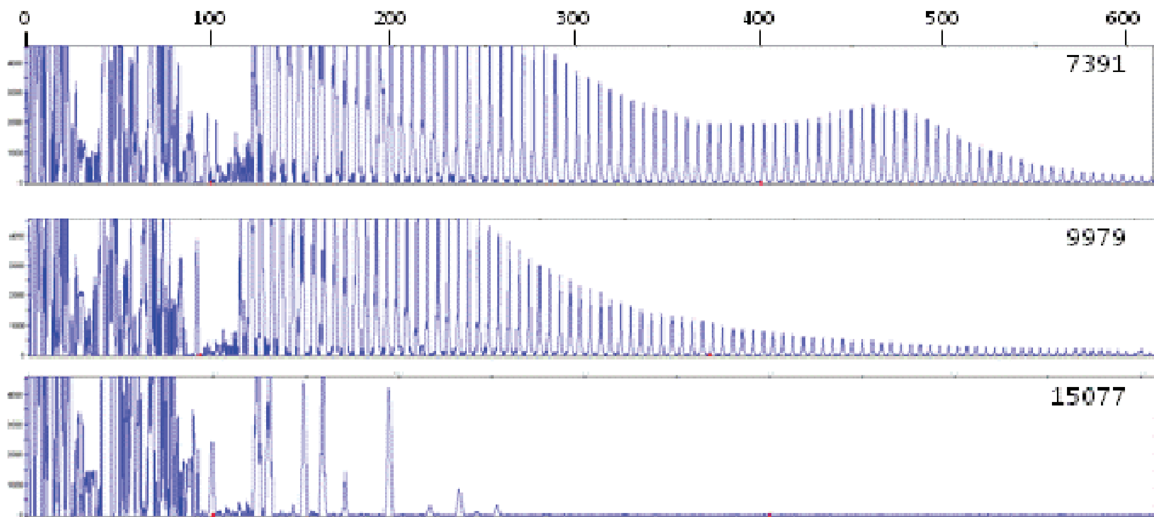

Figure 2. *C9ORF72* repeat-primed PCR assay in clinical AD patients. PCR products of *C9ORF72* repeat-primed PCR reactions separated on an ABI3730 DNA Analyzer and visualized by GENEMAPPER software. Electropherograms are zoomed to 4,000 relative fluorescence units to show stutter amplification. The two clinical AD patients with expanded repeats (7391 and 9979) and one non-carrier (15077) are shown. Note the strong amplification in patient 7391 around ~470bp, suggesting a large proportion of cells carrying approximately 60 GGGGCC repeats, in addition to a smaller population of cells carrying longer repeat expansions.

Table 5. Clinical characteristics of EOAD and FTD gene mutation carriers

Patient ID	Mutation	Sex	Age at onset (years)	Family history of dementia	# relatives with AD or memory problems	First clinical symptom(s)
506	*APP* p.Thr714Ile	M	41	Yes	4	Memory loss
11323	*PSEN1* p.Asn135Ser	F	34	Yes	2	Memory loss
9018	*PSEN1* p.Met146Leu	F	41	Yes	2	Memory and language
711	*PSEN1* p.Gly206Ala	M	52	Yes	2	Memory loss
15066	*PSEN1* p.Gly206Ala	M	53	Yes	4	Memory loss
11967	*PSEN1* p.Gly206Ala	F	63	No	0	Memory loss and language
10232	*PSEN1* p.Pro218Leu	F	55	Yes	1	Memory and vision loss
15408	*PSEN1* p.Ile238Met	M	60	Yes	4	Memory and vision loss
14915	*PSEN2* p.Ser130Leu	F	52	No	0	Memory loss
8120	*PSEN2* p.Met174Val	F	45	Yes	1	Memory loss
15229	*PSEN2* p.Phe183Ser	F	46	Adopted	unknown	Memory loss
14330	*GRN* p.Arg198Glyfs19X	M	62	No	0	Memory loss
15625	*MAPT* p.Arg406Trp	M	50	Yes	5	Memory loss
9979	*C9ORF72* repeat expansion	M	68	Yes	1	Memory loss
7391	*C9ORF72* repeat expansion	F	69	No	0	Memory and gait loss

APP, amyloid precursor protein, *PSEN1*, presenilin 1, *PSEN2*, presenilin 2, *GRN*, progranulin, *MAPT*, microtubule associated protein tau, *C9ORF72*, chromosome 9 open reading frame 72.

and one patient was adopted. The initial symptom always included memory loss which is why the clinical diagnosis of probable AD was made. None of the patients carrying mutations that typically cause FTD (*MAPT, GRN, C9ORF72*) presented with personality change or aphasia. Interestingly, 4 of the *PSEN1* patients had a focal onset, two with language difficulty and two with visual impairment. In the Alzheimer disease Mutation Database (www.molgen.ua.ac.be/ADMutations) [33] only one *PSEN1* mutation is reported to have progressive non-fluent

aphasia and none with the so called visual variant of AD [34]. Two of the patients were Hispanic and carried the known p.Gly206Ala mutation which was previously reported as a founder mutation in the Caribbean Hispanic population, suggesting these patients may be part of this extensive founder family [35].

Discussion

We report on the genetic characterization of a consecutive cohort of 227 clinical AD patients with an onset of dementia before 70 years of age ascertained at Mayo Clinic Florida. Despite the high proportion of AD patients with an early age at onset (63% with onset before 65 years) and a positive family history in more than half of the patients (56%), only 4.8% of our study cohort (11 patients) carried pathogenic mutations in the *APP* and *PSEN* genes. This low mutation frequency is similar to studies performed in the Danish population [36]; however other groups reported mutation frequencies of 22% [31] or even 71% [37]. The autosomal dominant pattern of inheritance in a large proportion of the patients included in the latter studies is likely to explain the significantly higher mutation frequency in these cohorts.

Two novel EOAD mutations were reported in this study. *PSEN1* p.Pro218Leu was identified in a familial AD patient with onset age at 55 years. This is the first mutation at *PSEN1* codon 218; however, several pathogenic mutations have been reported at the flanking codons 217 and 219, supporting the pathogenic nature of this mutation (www.molgen.ua.ac.be/ADMutations) [33]. We also observed a novel mutation in *PSEN2*. Since its discovery in 1995 as a causal EOAD gene, only 13 pathogenic *PSEN2* mutations have been reported in a total of 22 families. We identified a p.Phe183Ser mutation in exon 6 of *PSEN2* in an EOAD patient with first symptoms at 46 years. Unfortunately, since this patient was adopted no information on family history could be provided and no samples for segregation studies could be obtained. However, the strong conservation of this amino acid and the fact that the corresponding amino acid in PSEN1 has previously been shown to carry the same pathogenic mutation (p.Phe177Ser) provides strong support for its pathogenic nature (Figure 1). In contrast to p.Phe183Ser, the pathogenic nature of 4 coding variants found in exon 4 of *PSEN2* in this study remains less clear. Most of the patients carrying these variants (86%) were homo- or heterozygote for *APOE* ε4 allele. What is more, one patient carrying PSEN2 p.Arg62His also carried the PSEN1 p.Gly206Ala mutation which is a relatively common pathogenic mutation. Due to the fact that N-terminal domain of PSEN2 is a non-conserved region among mammalian species, it is more likely that the observed sequence variations are polymorphisms associated with increased risk of AD, rather than pathogenic mutations [38].

Based on previous findings that AD and FTD often share features that may lead to clinical misdiagnosis, we also determined the presence of common FTD gene mutations in our cohort of patients diagnosed as probable AD. For the *MAPT* gene, we specifically focused our analysis on the p.Arg406Trp mutation, previously reported to present with clinical AD in several families [39-42]. This mutation was identified in one AD patient (0.4%) with a strong family history of dementia. Using a GRN ELISA assay we further identified one AD patient (0.4%) with low plasma GRN levels which was found to carry the c.592_593delAG (p.Arg198Glyfs19X) mutation in *GRN*. This finding confirms previous studies reporting *GRN* loss-of-function mutations in clinically diagnosed AD patients [43-47]. Finally, we identified two unrelated patients carrying an expansion of the GGGGCC hexanucleotide repeat in the non-coding region of the newly discovered gene *C9ORF72*. Repeat-primed PCR analyses using DNA extracted from blood showed a predominant population of cells with approximately ~60 repeats for one patient, and a typical amplification pattern suggesting hundreds to even thousands of GGGGCC repeats for the other patient. The minimal repeat-size required for pathogenicity has not yet been established [48]; however, the maximum length observed in healthy control individuals is currently around 30 repeats, suggesting that both patients carry pathogenic repeats. Moreover, based on other non-coding repeat expansion disorders it is expected that repeat lengths may vary among different tissues within the same individual, and the repeat length in brain tissue may be significantly longer in both patients [49-51].

Recent reports showed high frequencies of *C9ORF72* repeat expansions in amyotrophic lateral sclerosis (ALS) and FTD patients, constituting approximately 40% and 25% of all ALS and FTD familial forms, respectively [22, 25, 48, 52]. In our clinical AD study cohort, the propor-

tion of *C9ORF72* expanded repeat carriers (0.9%) is comparable to that recently published by Majounie et al. (2012) [53], which reported *C9ORF72* repeat expansions in 6 out of 771 clinical AD patients. In their study, pathological examination in two repeat carriers showed FTLD-TDP pathology, suggesting that that these subjects had amnestic FTD that was misdiagnosed as probable AD. Autopsy confirmation was not available for the two *C9ORF72* repeat expansion carriers observed in our study; however, in a recent clinicopathological study of FTLD-TDP patients carrying *C9ORF72* repeat expansions from the Mayo Clinic brain bank, several patients were found to carry an ante-mortem diagnosis of Alzheimer-type dementia supporting the idea that misdiagnosis may be a consistent feature across probable AD populations [54]. Interestingly, despite the fact that we included a large proportion of young AD patients in our analyses, both *C9ORF72* repeat expansion carriers were among the oldest patients included in our study with onset ages of 68 and 69 years, respectively. Majounie et al. studied AD patients with onset ages ranging from 60-97 years and only identified expansion carriers with onset ages between 61-71 years, suggesting that this age range may be a characteristic feature of *C9ORF72* repeat expansion carriers with a predominant amnestic syndrome.

In summary, in addition to mutations in the known EOAD genes, we identified pathogenic mutations in all common genes related to FTD (*GRN*, *MAPT* and *C9ORF72)* in our cohort of patients diagnosed with clinical probable AD. Based on these findings, we believe that AD and FTD can be easily and frequently misdiagnosed as a result of overlapping clinical symptoms. Another reason of misdiagnosis is that the clinical presentation of AD and FTD may be atypical, especially in patients with an early disease onset. Our findings suggest that in clinically diagnosed AD patients, genetic analyses should include not only the well-established AD genes such as *APP*, *PSEN1* and *PSEN2* but also genes that are usually associated with FTD. Finally, we emphasize the fact that the low overall frequency of mutation carriers in our study cohort indicates that further genetic factors associated with EOAD must exist. Next-generation technologies such as exome-sequencing and whole-genome sequencing in specific AD families or extended series of well-characterized unrelated AD patients are likely to uncover novel AD genes in the near future.

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Conflict of interest

Rosa Rademakers and Mariely DeJesus-Hernandez have a patent pending on the discovery of the hexanucleotide repeat expansion in the *C9ORF72* gene.

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