C9orf72 Hexanucleotide Repeat Expansions in Clinical Alzheimer Disease

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**Importance:** Hexanucleotide repeat expansions in the chromosome 9 open reading frame 72 (C9orf72) gene underlie a significant fraction of frontotemporal dementia and amyotrophic lateral sclerosis.

**Objective:** To investigate the frequency of C9orf72 repeat expansions in clinically diagnosed late-onset Alzheimer disease (AD).

**Design, Setting, and Patients:** This case-control study genotyped the C9orf72 repeat expansion in 872 unrelated familial AD cases and 888 control subjects recruited as part of the National Institute on Aging Late-Onset Alzheimer Disease Family Study cohort, a multisite collaboration studying 1000 families with 2 or more individuals clinically diagnosed as having late-onset AD.

**Main Outcomes and Measures:** We determined the presence or absence of the C9orf72 repeat expansion by repeat-primed polymerase chain reaction, the length of the longest nonexpanded allele, segregation of the genotype with disease, and clinical features of repeat expansion carriers.

**Results:** Three families showed large C9orf72 hexanucleotide repeat expansions. Two additional families carried more than 30 repeats. Segregation with disease could be demonstrated in 3 families. One affected expansion carrier had neuropathology compatible with AD. In the National Institute on Aging Late-Onset Alzheimer Disease Family Study series, the C9orf72 repeat expansions constituted the second most common pathogenic mutation, just behind the PSEN1 A79V mutation, highlighting the heterogeneity of clinical presentations associated with repeat expansions.

**Conclusions and Relevance:** C9orf72 repeat expansions explain a small proportion of patients with a clinical presentation indistinguishable from AD, and they highlight the necessity of screening frontotemporal dementia genes in clinical AD cases with strong family history.

related AD cases and 888 unrelated control subjects and investigated whether expanded repeats were associated with age at dementia onset. Furthermore, we addressed whether a larger, but nonexpanded, GGGGCC allele was associated with the risk for AD or age at onset.

### METHODS

**PATIENTS**

Individuals from 872 unrelated families with at least 2 individuals affected by AD and 888 unrelated unaffected control subjects from the National Institute on Aging Late-Onset Alzheimer Disease (NIA-LOAD) Family Study were included. All AD cases had been diagnosed as having dementia of the Alzheimer type using criteria equivalent to the National Institute of Neurological and Communication Disorders and Stroke–Alzheimer Disease and Related Disorders Association for probable AD. All patients had a family history of AD but not other types of dementia or other neurodegenerative diseases. Probands were required to have a diagnosis of definite or probable late-onset AD (>60 years) and a sibling with definite, probable, or possible late-onset AD with a similar age at onset. A third biologically related family member (first, second, or third degree) was also required, regardless of affected status. If unaффected, this individual had to be 60 years of age or older, but 50 years of age or older if diagnosed as having late-onset AD or mild cognitive impairment. Within each pedigree, we selected a single individual to screen by identifying the youngest affected family member with the most definitive diagnosis (ie, individuals with autopsy confirmation were chosen over those with clinical diagnosis only). A summary of the demographics of all subjects is shown in Table 1. Written informed consent was obtained from all participants, and the study was approved by local institutional review board committees.

### GENETIC ANALYSIS

**Repeat-Primed Polymerase Chain Reaction**

The presence of the expanded hexanucleotide repeat and the number of repeat units in the longest allele were determined using previously reported methods for repeat-primed polymerase chain reaction (PCR) and fluorescence-based fragment size analysis. Briefly, repeat-primed PCR was performed in a total reaction volume of 28 μL containing 100 ng genomic DNA, 1x FastStart PCR Master Mix (Roche Applied Science), 3.5% dimethyl sulfoxide, 1x Q solution (Qiagen), and 0.18 mM deaza-dGTP. Products were run on a 2% agarose gel for visual inspection and then analyzed for fragment size determination, as described for repeat-primed PCR. Based on our analysis, cross-repeat PCR can amplify alleles with 35 or fewer GGGGCC repeats but not the 700 to 1600 repeats reported for pathologic expansions. To investigate repeat expansion segregation, we also genotyped all family members (n = 491) from every pedigree with 10 or more hexanucleotide repeats.

**Southern Blot**

Southern blot hybridization analysis was conducted using previously described methods and probe sequences to estimate the number of repeat units in expansion carriers.

### RISK HAPLOTYPE GENOTYPING

We also analyzed all repeat expansion carriers for the 24 single nucleotide polymorphism (SNP) at-risk haplotypes that have been associated with pathologically expanded C9orf72 repeats. All samples were genotyped with the Illumina Human 610 Beadchip, with direct genotyping of all analyzed SNPs. Stringent quality control criteria were applied to remove low-quality SNPs. We used the entire NIA-LOAD genomewide association study data set and the HapMap CEU population as reference populations. Mach software was used to phase the 24 SNPs.

### STATISTICAL AND BIOINFORMATIC ANALYSES

A linear regression model (SAS Institute Inc) was used to test whether the number of GGGGCC repeats was associated with the risk for AD, including age, sex, and apolipoprotein E (APOE) genotype as covariates. Association with age at onset was carried out using the Kaplan-Meier method and tested for significant differences using a Cox proportional hazards model (proc PHREG, SAS Institute Inc) that included sex and APOE status.

### RESULTS

**C9orf72 Repeat Expansion Frequency and Segregation in Alzheimer Disease Families**

We screened 872 unrelated familial AD cases and 888 unrelated control subjects for expansions of the hexanucleotide repeat in C9orf72. Five white individuals with a clinical diagnosis of AD (0.57%) and 1 normal control subject...
(age, 73 years) showed an abnormal expansion with repeat-primed PCR, defined as more than 30 repeats with 6 base-pair periodicity and the expected decrementing sawtooth pattern (fIFigure). To better clarify the number of repeats in these patients, we attempted to perform PCR across the GGGGCC repeat. However, in all 6 individuals, only a single peak representing the normal size allele was obtained. Southern blot of AD probands showed expansions of 1200 to 1300 GGGGCC repeats for families 1, 2, and 3, but only 35 to 100 repeat units in families 4 and 5 (data not shown). Regardless of repeat size, all 5 AD cases were found to carry the at-risk haplotype associated with pathologic repeat expansions in all patients with FTD and ALS reported to date (eTable 1). Results from all 5 of these patients were negative for the most common mutations in APP, PSEN1, PSEN2, GRN, and MAPT.

Pedigrees for the 5 families with abnormal (>30) C9orf72 repeats are shown in Figure 1 and clinically summarized in Table 2. All additional affected individuals had been diagnosed as having probable AD based on the National Institute of Neurological and Communication Disorders and Stroke–Alzheimer Disease and Related Disorders Association criteria. DNA for additional family members was available for all but family 1. Family 2 showed complete segregation of the large repeat expansion and risk haplotype with disease status (Figure 1 and eTable 2). Family 3, in which all the affected individuals were APOE 3/3 homozygous, showed segregation of the expanded repeat and risk haplotype except for a single individual who developed dementia with only 7 repeat units. Additionally, this individual did not carry the risk haplotype and had dementia onset at a later age than relatives with repeat expansions. In families 4 and 5 (repeat expansions between 35 and 100 repeats), the characteristic repeat-primed chromatogram pattern and the risk haplotype segregated perfectly with disease status. Overall, in these 5 families, 10 of 11 affected individuals for whom DNA was available carried abnormal GGGGCC expansions and the risk haplotype. It is important to note that all the individuals with more than 30 repeats also carried the risk haplotype.

The age at onset for individuals with more than 30 repeats was earlier than cases with normal repeat alleles (mean [SD], 65.6 [5.5] vs 71.4 [6.8] years; P = .04, Figure 2). This association retained statistical significance even after APOE genotypes were included in the model (P = .03).

### CLINICAL CASE AND NEUROPATHOLOGY DESCRIPTIONS

The proband of family 1 (1200-1300 repeats), an APOE 2/3 carrier, was diagnosed as having dementia, Alzheimer type, at age 73 years. Formal neurocognitive testing was not available, but prominent memory loss with repetitive questioning and wandering behavior had developed by age 78 years. On death 12 years after diagnosis, a brain autopsy was carried out by the Brain Bank at McLean Hospital in Belmont, Massachusetts, in 1999. Microscopic examination found extensive plaque and tangle pathology. Neurofibrillary tangles and neuritic plaques (>40 plaques per 100X field) were present throughout the neocortex, hippocampus, amygdala, and nucleus basalis of Meynert. Scattered neocortical Lewy bodies were seen. Severe neuronal loss in the substantia nigra pars compacta was accompanied by a few Lewy bodies. This case would be diagnosed as having NIA-Reagan high likelihood criteria for AD and coexisting neocortical-predominant Lewy body dementia using current criteria. Microscopic examination found extensive plaque and tangle pathology. Neurofibrillary tangles and neuritic plaques (>40 plaques per 100X field) were present throughout the neocortex, hippocampus, amygdala, and nucleus basalis of Meynert. Scattered neocortical Lewy bodies were seen. Severe neuronal loss in the substantia nigra pars compacta was accompanied by a few Lewy bodies. This case would be diagnosed as having NIA-Reagan high likelihood criteria for AD and coexisting neocortical-predominant Lewy body dementia using current criteria. Tissue was no longer available to carry out additional relevant staining (eg, tau, ubiquitin, TDP-43, or p62), preventing an assessment for possible FTD pathology.

Aside from age at symptom onset and meeting clinical criteria for probable AD, limited clinical information was available for the remaining index cases. The proband of family 2 (1200-1300 repeats) was diagnosed clinically as having probable AD at the age of 71 years, 3 years after disorientation and memory loss began. By the time of study inclusion at age 74 years, the patient was nonverbal and too impaired to participate in cognitive testing. The proband of family 3 (1200-1300 repeats) was diagnosed as having probable AD at the age of 60 years. Three years later, the Clinical Dementia Rating score was 0.5, which progressed over 3 years to 1.0. Review of limited caregiver re-
ports suggested that difficulties with disinhibition and anxiety were out of proportion to memory impairment. No additional clinical information was available for members of families 4 and 5 (35-100 repeats in each).

ASSOCIATIONS OF C9orf72 REPEAT LENGTH IN ALZHEIMER DISEASE

Previous studies in ALS and FTD have clearly demonstrated that C9orf72 repeat expansions are causative for disease. However, the minimum repeat number required for disease has not been established. Furthermore, it is not known whether higher repeat numbers (but still within the normal range) are associated with the risk for ALS or FTD. To address this question in AD, we compared the longest nonexpanded allele in cases and control subjects. The average longest allele was not statistically different from control subjects (mean [SD], 6.5 [4.1] vs 4.48 [3.7] repeat units; \( P = .10 \)), and the distribution of longest allele lengths were similar. Thus within the normal range, higher repeat numbers did not appear to be a risk factor for AD in this population. Furthermore, we found no association between the length of the longest nonexpanded allele and age at onset (\( P = .52 \)), nor evidence for an interaction with APOE genotype.

COMPARISON OF THE C9orf72 REPEAT EXPANSION FREQUENCY WITH OTHER PATHOGENIC GENE MUTATIONS

In a previous study, we sequenced APP, PSEN1, PSEN2, MAPT, and GRN genes in a discovery series comprising 439 cases included in this study. The most common pathogenic mutation identified by sequencing in the discovery series (PSEN1 A79V) was then genotyped in the entire cohort (follow-up series). Overall, the A79V mutation was found in 4 of the 872 cases (0.46%) compared with the 5 pedigrees where abnormal C9orf72 repeat expansions were found. Furthermore, we analyzed the overall frequency of AD gene mutations (APP, PSEN1, and PSEN2) vs FTD gene mutations (MAPT, GRN, and C9orf72) and found that 1.82% of probands carried a pathogenic, or very likely pathogenic, mutation in APP, PSEN1, and PSEN2, while a slightly larger number (1.94%) had mutations in MAPT, GRN, or C9orf72.

This study assessed C9orf72 hexanucleotide expansions in familial late-onset AD cases and normal control subjects, identifying 5 AD families carrying abnormal C9orf72 hexanucleotide repeat expansions. This frequency is very

<table>
<thead>
<tr>
<th>C9orf72 Repeat Expansion</th>
<th>Affected With AD</th>
<th>Unaffected</th>
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<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Overall</td>
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</tr>
<tr>
<td>Age, mean (SD) [range], y</td>
<td>65.3 (4.9) [60-73]</td>
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<tr>
<td>Age, y</td>
<td>65, 68</td>
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</tr>
<tr>
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<td>1</td>
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<tr>
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<tr>
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<tr>
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<tr>
<td>Age, y</td>
<td>67, 70</td>
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</tr>
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</table>

Abbreviations: AD, Alzheimer disease; C9orf72, chromosome 9 open reading frame 72; NA, not applicable.

For patients with AD, age refers to age at symptom onset; for unaffected individuals, age refers to the age at last assessment.

Table 2. Segregation Analysis of Pedigrees With Repeat Expansions

Figure 2. C9orf72 repeat expansions correlate with an earlier age at onset. Age at onset was analyzed for association with repeat expansion genotypes by the Kaplan-Meier method and tested for significant differences using a proportional hazards model (proc PHREG; SAS Institute Inc). Repeat expansion carriers show an earlier age at onset than noncarriers (mean [SD], 68.25 [5.8] vs 71.4 [6.8] years; \( P = .04 \)).
similar to that found in an independent AD series, but significantly lower than in FTD or ALS. Three families with clinical AD (0.34%) were found to have repeat expansions in the range reported for FTD and ALS (>1 000). In a previous report documenting expansions in clinically diagnosed AD, reevaluation of autopsy material demonstrated FTD pathology and suggested that AD cases with C9orf72 repeat expansions represent amnestic variants of FTD. \(^{9,14,15,27,28}\) We were unable to perform an equivalent analysis because autopsies had not been performed or tissue was no longer available. Therefore, even in the proband from family 1, where the neuropathology showed coexisting AD and Lewy body dementia pathology, we cannot rule out the possibility that the families identified in this study also represented amnestic presentations of FTD rather than AD. It is notable that of the many C9orf72 FTD cases reported, several showed concurrent AD pathology \(^{9,14,15,27,28}\) and an additional expansion carrier with FTD+ALS demonstrated enough plaque and tangle pathology to meet diagnostic criteria for AD. \(^{27}\) These cases suggest that some individuals with C9orf72 repeat expansions could present with clinical symptoms of AD and have a high enough burden of AD neuropathology that biomarker analysis (cerebrospinal tau or amyloid, and/or Pittsburgh Compound B–positron emission tomography neuroimaging) would also support an AD diagnosis. This hypothesis is supported by 3 recently reported individuals with early-onset AD, cerebrospinal fluid profiles typical of AD, \(^{27}\) who were found to carry C9orf72 repeat expansions. In this setting, the correct diagnosis (amnestic FTD) would presumably only be reached by neuropathologic studies or genetic testing. Our cases and previous studies reinforce the heterogeneous clinical and neuropathologic presentations of C9orf72 repeat expansions (ALS, frontotemporal lobar degeneration, frontotemporal lobar degeneration–ALS, and clinical AD).

We also identified 2 families carrying smaller, but abnormal repeats (>35, but <100 units). Despite segregating with disease status, it remains unclear whether these smaller repeat expansions cause disease, increase the risk for dementia, or are incidental. Future studies correlating quantified repeat sizes with disease status will be required to answer this question.

Although the frequency of large C9orf72 repeat expansions was low in our cohort, it was the second most common pathogenic mutation (3 of 872), just behind PSEN1 A79V (4 of 872). In addition, mutations in FTD genes were as common as mutations in AD genes (1.94% vs 1.87%). Our results confirm that the clinical phenotype of mutations in FTD genes, including GRN, MAPT, and C9orf72, can be clinically indistinguishable from typical AD. \(^{30-34}\) This fact has important implications for clinicians, who should consider both FTD and AD genes when evaluating families with strong histories of AD.

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