C9orf72 hexanucleotide repeat length in older population: normal variation and effects on cognition

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Karri Kaivola [a,*], Anna Kiviharju [a], Lilja Jansson [a], Ville Rantalainen [b],
Johan G. Eriksson [c,d,e], Timo E. Strandberg [f,g], Hannu Laaksovirta [a], Alan E. Renton [h],
Bryan J. Traynor [i], Liisa Myllykangas [j], Pentti J. Tienari [a]

[a] *Molecular Neurology, Research Programs Unit, Department of Neurology, University of Helsinki, Helsinki University Hospital, Helsinki, Finland*
[b] *Department of Psychology and Logopedics, University of Helsinki, Helsinki, Finland*
[c] *Folkhälsan Research Center, Helsinki, Finland*
[d] *Department of General Practice and Primary Health Care, University of Helsinki, Helsinki University Hospital, Unit of General Practice, Helsinki, Finland*
[e] *Department of General Practice and Primary Health Care, National Institute for Health and Welfare, Helsinki, Finland*
[f] *Centre for Life Course Health Research, University of Oulu, Oulu, Finland*
[g] *University of Helsinki, Helsinki University Hospital, Helsinki, Finland*
[h] *Department of Neuroscience, Ronald M. Loeb Center for Alzheimer's Disease, Icahn School of Medicine at Mount Sinai, New York, NY, USA*
[i] *Laboratory of Neurogenetics, National Institutes on Aging, NIH, Bethesda, MD, USA*
[j] *Department of Pathology, University of Helsinki and HUSLAB, Helsinki University Hospital, Helsinki, Finland*

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ABSTRACT

The hexanucleotide repeat expansion in *C9orf72* is a common cause of amyotrophic lateral sclerosis/frontotemporal dementia and also rarely found in other psychiatric and neurodegenerative conditions. Alleles with >30 repeats are often considered an expansion, but the pathogenic repeat length threshold is still unclear. It is also unclear whether intermediate repeat length alleles (often defined either as 7–30 or 20–30 repeats) have clinically significant effects. We determined the *C9orf72* repeat length distribution in 3142 older Finns (aged 60–104 years). The longest nonexpanded allele was 45 repeats. We found 7–45 repeats in 1036/3142 (33%) individuals, 20–45 repeats in 56/3142 (1.8%), 30–45 repeats in 12/3142 (0.38%), and expansion (>45 repeats) in 6/3142 (0.19%). There was no apparent clustering of neurodegenerative or psychiatric diseases in individuals with 30–45 repeats indicating that 30–45 repeats are not pathogenic. None of the 6 expansion carriers had a diagnosis of amyotrophic lateral sclerosis/frontotemporal dementia but 4 had a diagnosis of a neurodegenerative or psychiatric disease. Intermediate length alleles (categorized as 7–45 and 20–45 repeats) did not associate with Alzheimer's disease or cognitive impairment.

1. Introduction

C9orf72 hexanucleotide repeat expansion is a major cause of sporadic and familial amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) (DeJesus-Hernandez et al., 2011; Renton et al., 2011), and it is particularly common in Finland (Majounie et al., 2012). Expansions can span up to several 1000 repeats, but the minimum length of pathogenic expansion is not known. Knowledge on the distribution of repeat lengths in the general population could help gain a better understanding of the threshold for pathogenicity. Perhaps the most widely used threshold for expansion is 30, defined in one of the original studies describing the *C9orf72* repeat (Renton et al., 2011). Determining a threshold for expansion is complicated by the difficulty of reliably determining repeat lengths, and thresholds reflect more the limitation of used methods than actual alteration in biologic function. Southern blot is considered to be the golden standard of repeat length estimation but because of its high demand of good quality DNA, cost, and work load, often other methods such as repeat-primed PCR (RP-PCR) are used.

Besides genuine expansions, "intermediate length" alleles (defined often as 7–30 or 20–30 repeats) have also been hypothesized to

* Corresponding author at: University of Helsinki, Research Programs Unit, Haartmaninkatu 8, P.O. Box 63 00014, Helsinki, Finland. Tel.: +358 50 522 3563; fax: +35 891 912 5610.

E-mail address: karri.kaivola@helsinki.fi (K. Kaivola).

predispose to neurodegenerative diseases. In luciferase reporter assay, intermediate alleles showed decreased *C9orf72* promoter activity as compared to short repeat alleles (Gijselinck et al., 2016). Moreover, adverse effects have been observed in a fly model with 30 repeats (Zhang et al., 2015), although the relevance of fly physiology to human pathophysiology in this context is unsure. As with expansion, the threshold for intermediate length repeats also varies in studies but allele length ≥ 7 has been shown to associate with the *C9orf72* founder haplotype in the European population (van der Zee et al., 2013). A recent review (Ng and Tan, 2017) summarized that most studies do not find association between intermediate length alleles and a variety of neurodegenerative diseases but intermediate length alleles might be associated with psychiatric symptoms. Most studies on intermediate length alleles focus on ALS/FTD spectrum but only few on Alzheimer's disease (AD) (Cacace et al., 2013; Harms et al., 2013; Jiao et al., 2013; Kohli et al., 2013; Xi et al., 2012).

In this study, we present *C9orf72* hexanucleotide repeat length distribution in 3142 Finns and study the association of intermediate repeat alleles with AD and cognitive impairment.

2. Methods

2.1. Cohorts

We studied the *C9orf72* hexanucleotide repeat length in 3161 individuals from 4 population-derived cohorts from the Helsinki region in Southern Finland. These were the Vantaa 85+ study (Tanskanen et al., 2017) (n = 469), Helsinki Birth cohort study (Barker et al., 2005; Eriksson et al., 2006; Kajantie et al., 2012; Lahti et al., 2014; Yliharsila et al., 2007) (n = 1651), Helsinki Businessmen study (Strandberg et al., 2016) (n = 666), and DEBATE study (Uusvaara et al., 2013) (n = 375). We have previously used the same cohorts to study the association of *TYROPB* deletion and cognitive impairment (Kaivola et al., 2018). Information on AD, other dementia diagnoses, mini mental state examination (MMSE) scores, or lack of dementia was assessed from clinical records, death certificates, registry information, or questionnaires. The source of information varied between cohorts. More detailed cohort descriptions and information on assessment of dementia are provided in the Supplementary Methods.

We studied the effect of intermediate repeat alleles in 3 settings: (1) individuals with AD versus no AD; (2) individuals with cognitive impairment (MMSE score ≤ 24) versus individuals with no cognitive impairment (MMSE score >24), and (3) individuals with cognitive impairment or any diagnosis of dementia versus nondemented controls. All controls were ≥ 75 years. Repeat lengths 7—45 and 20—45 were used as the definition of intermediate length alleles.

2.2. Genetic analyses

We determined repeat lengths with RP-PCR as previously described (Renton et al., 2011) with minor changes to the protocol (Supplementary methods). Because it may be difficult to distinguish between an expansion and a long repeat in RP-PCR, we confirmed alleles with repeat length ≥ 20 and all putative expansions with over-the-repeat PCR. Samples with more than 30 repeats and the typical sawtooth pattern in RP-PCR, which only produced the smaller amplicon in over-the-repeat PCR, were categorized as expansions (Supplementary Fig. 1).

The smallest allele is here denoted as 2—3 repeats because 2 and 3 repeat alleles cannot be distinguished by RP-PCR. RP-PCR results may be inaccurate in distinguishing heterozygosity and homozygosity in certain allele combinations (especially 2—3/5 from 5/5 and 5/8 from 8/8). This can slightly increase the proportion of heterozygotes at the expense of homozygotes. Considering this uncertainty, we did not

analyze cognitive measures separately in individuals homozygous for the intermediate alleles. There were no samples homozygous for the 20—45 repeat alleles in our cohorts. *APOE* was genotyped as previously described (Myllykangas et al., 1999).

2.3. Statistics

We used logistic regression to test for association between intermediate repeats in all 3 settings and used *APOE* $\varepsilon 4$ carriership, age, and sex as covariates. We applied Bonferroni correction to take account of multiple test settings and set the statistical significance to $p = 0.05/3 = 0.017$. We used Kruskal-Wallis H test to determine if repeat length distributions differed between our 4 cohorts. All analyses were conducted with IBM SPSS statistics v.24 (IBM Corp. Released, 2016. IBM SPSS Statistics for Windows, Version 24.0. IBM Corp., Armonk, NY, USA).

Assuming AD prevalence of 10% in Finnish individuals aged over 75 years (Rahkonen et al., 2003), intermediate allele (≥ 7 repeats) frequency of 17% based on our initial results, case-control ratio of 1:4, type 1 error rate 0.05 and relative risk of 1.5 with dominant effect, 201 cases were required for 80% power (Purcell et al., 2003) (Genetic power calculator, http://zzz.bwh.harvard.edu/gpc/ accessed 4.6.2018). From the 4 cohorts, we identified 226 individuals with AD, 432 with MMSE ≤ 24, and 613 with any diagnosis of dementia or cognitive impairment.

2.4. Ethics

The study was approved by the Coordinating Ethics Committee of the Helsinki University Central Hospital. The Vantaa 85+ study was also approved by the Ethics Committee of the Health Centre of the City of Vantaa.

3. Results

3.1. Repeat length distribution

We successfully determined repeat lengths in 3142/3161 (99.4%) individuals. There was no statistically significant difference in the repeat length distributions between cohorts [H (3) = 2.129, $p = 0.55$]. Repeat length distribution of all alleles is shown in Fig. 1. The repeat length distribution showed peaks in repeat numbers 2—3, 5, 8, and 10. The longest nonexpanded allele we could characterize with repeat-primed PCR and amplify with over-the-repeat PCR was 45 repeats. The longer allele was in the range of 7—45 repeats in 1036/3142 (33%) individuals, 20—45 in 56/3142 (1.8%), 30—45 in 12/3142 (0.38%), and expansion (>45 repeats) in 6/3142 (0.19%). All expansion carriers were from the largest (HBCS) cohort.

Interestingly, repeat lengths ≥ 20 were more common in Finland than reported elsewhere in previous large studies. To compare the frequency of ≥ 20 repeat lengths, we searched PUBMED for large population based (>1000 individuals) and case-control studies (>1000 controls) with *C9orf72* repeat length distribution available. Besides the original articles describing the repeat, we identified 9 articles (Beck et al., 2013, Cacace et al., 2013, Fahey et al., 2014, He et al., 2015, Kohli et al., 2013, Nuytemans et al., 2013, Rutherford et al., 2012, Theuns et al., 2014, van der Zee et al., 2013) but only 4 had enough detailed information on ≥ 20 repeats for comparison. In these 4 studies, the prevalence of ≥ 20 repeats was significantly lower than in Finland (all allelic frequencies $<0.52\%$ and all $p < 0.019$, Fisher's exact test) (Table 1). Of the 9 studies, only 2 found expansion carriers in their control groups with carrier frequency of 1/5748 ($p = 0.0095$ vs. our Finnish sample, Fisher's exact test) (Theuns et al., 2014) and 11/7579 ($p = 0.60$ vs. FIN) (Beck et al., 2013).

Fig. 1. Repeat length distribution of all alleles in 3142 Finns. "Exp" denotes expansion.

3.2. Intermediate repeat length alleles, Alzheimer's disease, and cognitive impairment

Number of individuals in each cohort with or without AD/cognitive impairment is shown in Supplementary Table 1.

3.2.1. Alzheimer's disease

We identified 226 individuals with AD (68–97 years, mean age 86, 58% female, 33% carried 7–45 repeats, 1.3% carried 20–45 repeats) and 893 controls (aged 75–101, mean age 83, 58% female, 33% carried 7–45 repeats, 1.3% carried 20–45 repeats). Intermediate repeat length alleles did not associate with AD when intermediate repeat threshold was ≥ 7 ($p = 0.90$) or ≥ 20 ($p = 0.96$). Age ($p = 3.8 \times 10^{-9}$, odds ratio [OR] 1.07, 95% confidence interval [CI] 1.05–1.10) and *APOE* $\varepsilon 4$ carriership ($p = 5.5 \times 10^{-10}$, OR 2.69, CI 1.97–3.68) were statistically significantly associated with AD but sex was not ($p = 0.45$).

3.2.2. MMSE scores

When individuals were divided based only on MMSE score into cases (MMSE score ≤ 24, n = 432, aged 61–99, mean age 85, 75% female, 27% carried 7–45 repeats, 1.6% carried 20–45 repeats) and controls (MMSE score ≥ 25, n = 719, aged 75–98, mean age 80, 38%

female, 33% carried 7–45 repeats, 1.3% carried 20–45 repeats), there was a nominally significant association between 7–45 repeats and better cognition ($p = 0.025$, OR 0.71 for cognitive impairment, CI 0.53–0.96), but the association did not remain statistically significant after Bonferroni correction. This association disappeared when repeat length ≥ 20 was used as threshold for intermediate repeat allele (20–45 repeats, $p = 0.72$). Age ($p = 5.0 \times 10^{-20}$, OR 1.1, CI 1.1–1.2), female sex (9.8×10^{-14}, OR 3.1, CI 2.3–4.1), and *APOE* $\varepsilon 4$ carriership ($p = 0.00078$, OR 1.7, CI 1.2–2.2) associated significantly with cognitive impairment.

3.2.3. Any diagnosis of dementia or cognitive impairment

We identified 613 cases (aged 62–101, mean age 86, 65% female, 29% carried 7–45 repeats and 1.3% carried 20–45 repeats) and 1115 controls (aged 75–101, mean age 83, 37% female, 34% carried 7–45 repeats and 1.5% carried 20–45 repeats). There was a nominally significant association between intermediate repeat lengths and protection from dementia or cognitive impairment ($p = 0.032$, OR 0.78, CI 0.62–0.98), but this result was not statistically significant after Bonferroni correction, and again, the association was lost when intermediate repeat threshold ≥ 20 was used (20–45 repeats, $p = 0.62$). Age ($p = 5.6 \times 10^{-18}$, OR 1.08, CI 1.06–1.1), female sex

Table 1
Comparison of frequency of ≥ 20 repeats and expansions in population based datasets or large (n > 1000) control samples from case-control studies

Population	Number of individuals	Number of ≥ 20 repeats (expansions excluded)	Allelic frequency of ≥ 20 repeats (%)	*p*-value versus our sample	Expansions in controls (n)	Reference
Finnish	3142	56	0.89		6	This study
British 1958 birth cohort	7577	64[a]	0.42	0.000051	11	(Beck et al., 2013)
Irish	1234	10	0.41	0.019	0	(Fahey et al., 2014)
European/Asian/North American/Australian	5886	61 (≥ 17 repeats)	0.52	0.0045	1	(Theuns et al., 2014)
North American	1444	11	0.38	0.0078	0	(Rutherford et al., 2012)

[a] Written personal communication from Professor Mead.

Table 2
Summary of individuals with ≥30 repeats

Individual number	Sex	Age at last information collection	Repeat length (shorter/longer allele)	Information on cognition
1	Female	91	2/30	MMSE score 26[b]
2	Male	70	2/31	MMSE score 29[b]
3	Female	72	2/31	No dementia diagnoses[b]
4	Male	85	5/31	MMSE score 28[b]
5	Male	70	2/32	MMSE score 30[b]
6	Male	81	2/34	MMSE score 29[b]
7	Female	89	5/36	MMSE score 21[b]
8	Male	71	2/40	MMSE score 30/No dementia or psychiatric diagnoses
9	Male	72	2/43	No dementia or psychiatric diagnoses
10	Female	77	2/45	MMSE score 29/No dementia or psychiatric diagnoses
11	Male	74	2/45	MMSE score 29/No dementia or psychiatric diagnoses
12	Female	73	8/45	MMSE score 29/No dementia or psychiatric diagnoses
13	Male	79	2/expansion	G20 (Parkinson disease, ICD-10), No psychiatric diagnoses.
14	Female	60[a]	2/expansion	300.40 (depressive neurosis, ICD-8), 300.88 (other neurosis, ICD-8). No dementia diagnoses.
15	Female	71	2/expansion	G31.0 (local brain atrophy, ICD-10). No psychiatric diagnoses.
16	Male	68[a]	5/expansion	No dementia or psychiatric diagnoses
17	Female	71	6/expansion	No dementia or psychiatric diagnoses
18	Female	74	8/expansion	291.20 (Alcoholic psychosis, ICD-8). No dementia diagnoses.

[a] Age of death, cause of death coronary artery disease.
[b] Psychiatric diagnoses were not available. RP-PCR chromatograms are provided in Supplementary Fig. 2.

($p = 1.1 \times 10^{-24}$, OR 3.0, CI 2.5—3.8), and *APOE* ε4 carriership ($p = 4.0 \times 10^{-7}$, OR 1.8, CI 1.4—2.2) associated significantly with dementia or cognitive impairment.

3.2.4. Neurodegenerative and psychiatric diagnoses

We did not find any evident clustering of neurodegenerative or psychiatric disease in the 12 (0.38%) individuals with 30—45 repeats (Table 2). There were 6 (0.19%) expansion carriers and none had diagnosis of ALS or FTD; 2 had died at ages of 60 and 68 years because of coronary artery disease. Of the 4 living individuals (aged 71—79 years), one had been diagnosed with Parkinson disease (G20, ICD-10) and another had a diagnosis of local brain atrophy (G31.0, ICD-10). A third had been diagnosed with "other alcoholic hallucinosis" (291.20, ICD-8) and a fourth with depressive neurosis and other neurosis (300.40 and 300.88, ICD-8). A summary of carriers of 30—45 repeats and expansions is shown in Table 2.

4. Discussion

The *C9orf72* hexanucleotide repeat expansion is a major cause of ALS/FTD; however, what repeat lengths are pathogenic is still unclear. Our data on 3142 older individuals show that 30—45 repeats are found in 0.38% (1 per 262) individuals, and they did not have any apparent clustering of neurodegenerative or psychiatric disease. Our data suggest that 30—45 repeat alleles should not be considered unequivocally pathogenic and other risk factors than the *C9orf72* expansion should be considered in patients with these alleles. The smallest expansions that segregate with combinations of ALS, FTD-ALS, and "FTLD or dementia" are reportedly ca. 55—100 repeats as measured by Southern blot (Gijselinck et al., 2016). However, an individual with 70 repeats without neurodegeneration at the age of 90 years has been reported (McGoldrick et al., 2018). More data are still needed for more precise estimation of the pathological threshold, which may actually turn out as a "gray zone" where disease penetrance is dependent on modulating cofactors.

Our data are in line with previous studies indicating a lack of major association between intermediate repeats and AD. Somewhat surprisingly, there was a nominally significant association between 7—45 repeat length alleles and better cognition. This association was not statistically significant in any of the individual cohorts and was only seen in the pooled data set. This can be a spurious association, but nevertheless, it supports the hypothesis that ≥7 repeat alleles do not predispose to cognitive impairment or dementia. Across all our cohorts, there was heterogeneity in sex and age distributions, variation in socioeconomic status, and educational history (one cohort consisted solely of men with high socioeconomic status), and there was no uniform way of defining cognition and dementia status, and this information was collected from several sources. Despite these limitations, we found a clear association between *APOE* ε4 genotype and AD ($p = 5.5 \times 10^{-10}$, OR 2.69), cognitive impairment ($p = 0.00078$, OR 1.7), and overall dementia status ($p = 4.0 \times 10^{-7}$, OR 1.8), which argues against a major bias in our definition of dementia. Furthermore, the Finnish national registers have been found to identify dementia cases accurately (Solomon et al., 2014).

None of the 6 expansion carriers we identified had a diagnosis of ALS or FTD but 4 had a diagnosis of a neurodegenerative or psychiatric disease. Two had died of coronary heart disease before the age of 70 years and others were under 80 years. Therefore, it is possible that some expansion carriers could have developed or may develop ALS/FTD in their later years.

Previous reports have shown that *C9orf72* expansion is especially common cause of ALS/FTD in Finland (Majounie et al., 2012). In the present study, the expansion frequency in older Finns (median age 77) was only slightly higher than in the UK 1958 birth cohort. However, this UK population was considerably younger (age ca. 54—55 years during publication in 2013) than our Finnish sample. The penetrance of the *C9orf72* expansion is known to be strongly age related; nearly complete penetrance was observed by 83 years in a cohort of 1147 ALS and FTD cases. The median age of onset varied somewhat with phenotype but was estimated to be around 58 years (Murphy et al., 2017). Therefore, it can be expected that the expansion frequency in the UK cohort will markedly decrease by time. In line with age-related penetrance, the expansions were unequally distributed in our cohorts; all Finnish expansion carriers were from the HBCS cohort, whose participants were the youngest of the 4 cohorts (born 1934—1944, and registry information available up till December 31 of 2013, Supplementary methods). If we restrict the analysis to this cohort only, expansion prevalence would be 6/1644 (0.36% or 1 in 274) in Finland.

Interestingly, we found that nonexpanded repeat lengths ≥20 are roughly twice as common in Finland than in other reported populations (Table 1). This finding may have a connection with the

high frequency of *C9orf72* expansion in Finland and raises the question whether the ≥20 repeat alleles could be prone to germline instability and expansion in the offspring. In this scenario, the larger intermediate alleles would form a pool from which new expansions can be derived, a mechanism shown in families with sporadic Huntington's disease and in Huntington's disease CAG repeat knock-in mice (Goldberg et al., 1993; Myers et al., 1993; Wheeler et al., 1999). Somatic instability (mosaicism) of the expansion has been previously demonstrated (Dols-Icardo et al., 2014; Fratta et al., 2015) and at least one family has been described, in which an unaffected 90-year-old father had a 70-repeat allele in blood (premutation), which was transmitted to 4 offspring, who all had expansion in blood DNA (McGoldrick et al., 2018). Whether the expansion was present in the father's sperm could not be tested. However, mosaicism with large expansions was demonstrated in other tissues; therefore, it is likely that the expansion was present in sperm. As more than half of the Finnish patients with ALS with the *C9orf72* expansion are sporadic (Majounie et al., 2012) (Laaksovirta et al. unpublished data), the spectrum of premutations warrants further research by genotyping sporadic *C9orf72* ALS cases' parents when possible. Another approach would be to genotype paired DNA samples from blood and gametes (Martorell et al., 2004), especially in carriers of the longer alleles.

Disclosure

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.neurobiolaging.2019.02.026.

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