The American Journal of Human Genetics

Supplemental Data

Jump from Pre-mutation to Pathologic Expansion in *C9orf72*

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Clinical Descriptions

None of the seven family members of PED25 have history of physical trauma, illegal drug abuse or pesticide exposure.

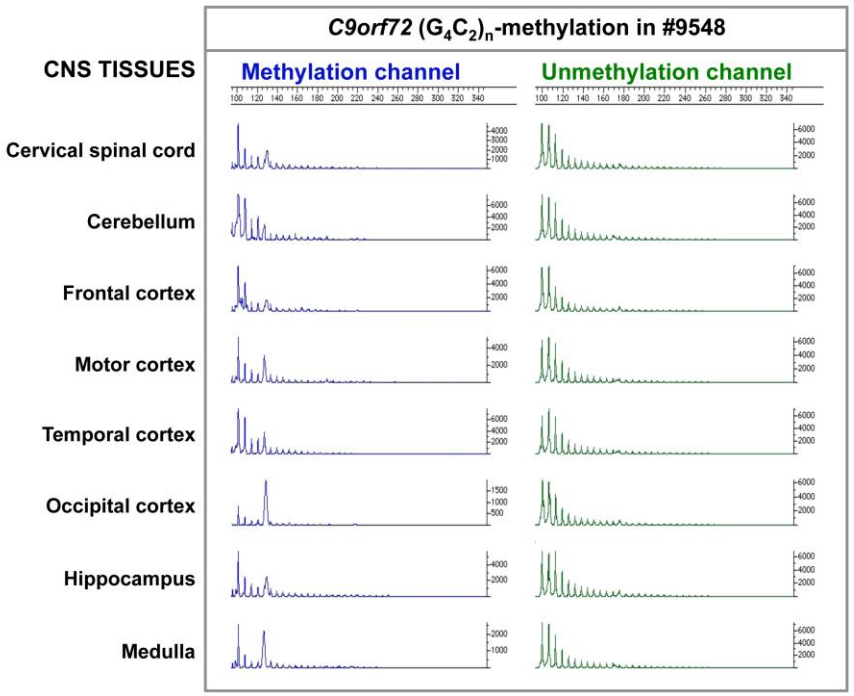
Both parents of the proband are alive without symptoms of ALS or FTLD, based on neurological assessments. The 90-year-old mother (#9685) has recent onset of mild memory loss. The 89-year-old father (#9686) has a medical history of chronic obstructive pulmonary disease and heart disease. He was an officer in the British and Canadian navy, "always mentally sharp" as reported by his children. He is still independent for all activities of daily living. His performance fell within the normal range in the Social Norm Questionnaire with a score of 16/22, but he noted some difficulty with short-term memory over the last year (at age 89), which was reflected in a MoCA score of 23/30, losing 2 points for mistakes with the clock drawing test and 5 points for delayed recall. Although the MoCA score of #9686 is within the range of mild cognitive impairment (18-26), it is still above the average (21) of the general elderly population of 70-80 years old [1]. His mild cognitive impairment might indicate early disease symptoms; however the MoCA score is still above the average for his age-group. Of note, 65% of Canadians older than 85 have cognitive impairment [2].

The proband (#9548) developed symptoms of bulbar onset at age 57 without any evidence of cognitive deficit on a Montreal Cognitive Assessment (MoCA) [3] (29/30). This individual had a college education and worked as an emergency unit clerk. The medical history included hypothyroidism, chronic obstructive pulmonary disease, cholecystectomy, incontinence, hysterectomy and a hernia. Individual #9548 smoked a pack of cigarettes per day for 37 years starting at age 14 and drank a glass of red wine daily. At age 59 individual #9548 passed away and autopsy results confirmed the diagnosis of ALS with pathological signs of early stage FTLD.

One of the four proband's siblings (#8665) also developed ALS at age 59 and passed away at age 62 (autopsy was not performed). Individual #8665 had lumbar onset ALS with primary symptoms in the upper limbs (bulbar symptoms developed at the end stage of the disease). At the time of diagnosis individual #8665 was completing an MBA degree, and MoCA score was within normal range (27/30), with one point missed for attention, orientation and delayed recall, respectively. The medical history was significant for bipolar affective disorder treated by Lithium. Individual #8665 had a history of heavy smoking (up to two packs daily) and drinking (two or more drinks daily).

Three other siblings (#9697, #9698 and #9707) remain asymptomatic at their current age (65, 65, and 51 years, respectively). They all completed a college education and worked professionally (e.g. in healthcare, human resources, or arts). All of them had smoking habits for 6-7 years (~5 cigarettes per week). They did not report any changes in their cognitive abilities, with the exception of #9698, who complained about memory issues, affecting daily activities. All three asymptomatic siblings underwent cognitive screening using MoCA and ALS Cognitive Behavioural Screen [4], showing performance within normal range.

A.



B.

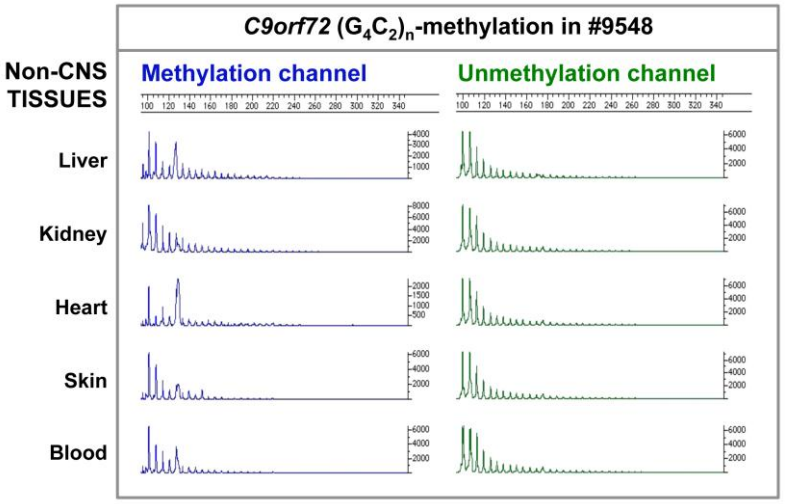


Figure S1. Chromatograms of the $(G_4C_2)_n$-methylation assay for #9548 in multiple autopsy tissues: (A) central nervous system (CNS) tissues (B) non-CNS tissues

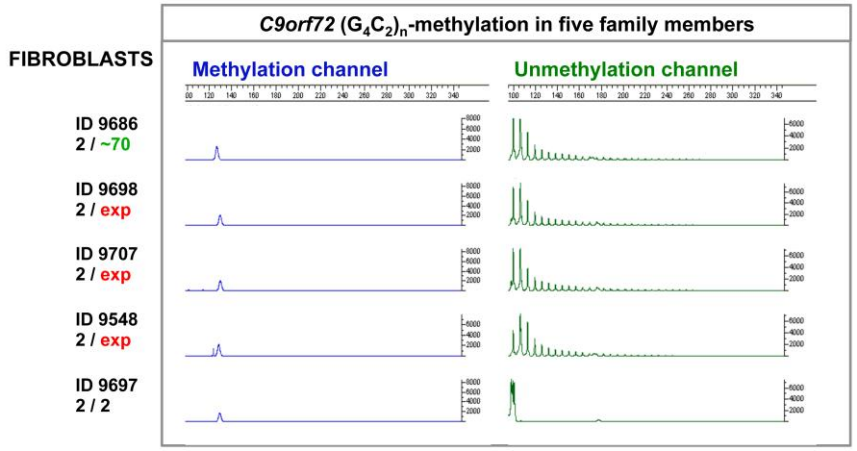


Figure S2. Chromatograms of the $(G_4C_2)_n$-methylation assay for PED25 in fibroblast samples

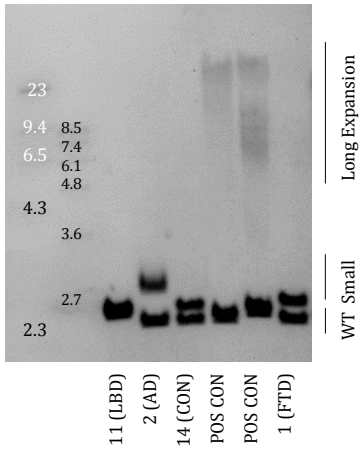


Figure S3. Southern blot results for subjects with small expansions or repeats in the long wild-type range.

Subjects are diagnosed with Lewy Body Dementia (LBD), Alzheimer's disease (AD), frontotemporal dementia (FTD), or they are unaffected (CON). Two positive controls are included with a long expansion on one allele and wild-type alleles of 2 and 11, respectively (POS CON). Case #2 has been described by Wojtas *et al.* [5]. Numbers correspond to Table S1.

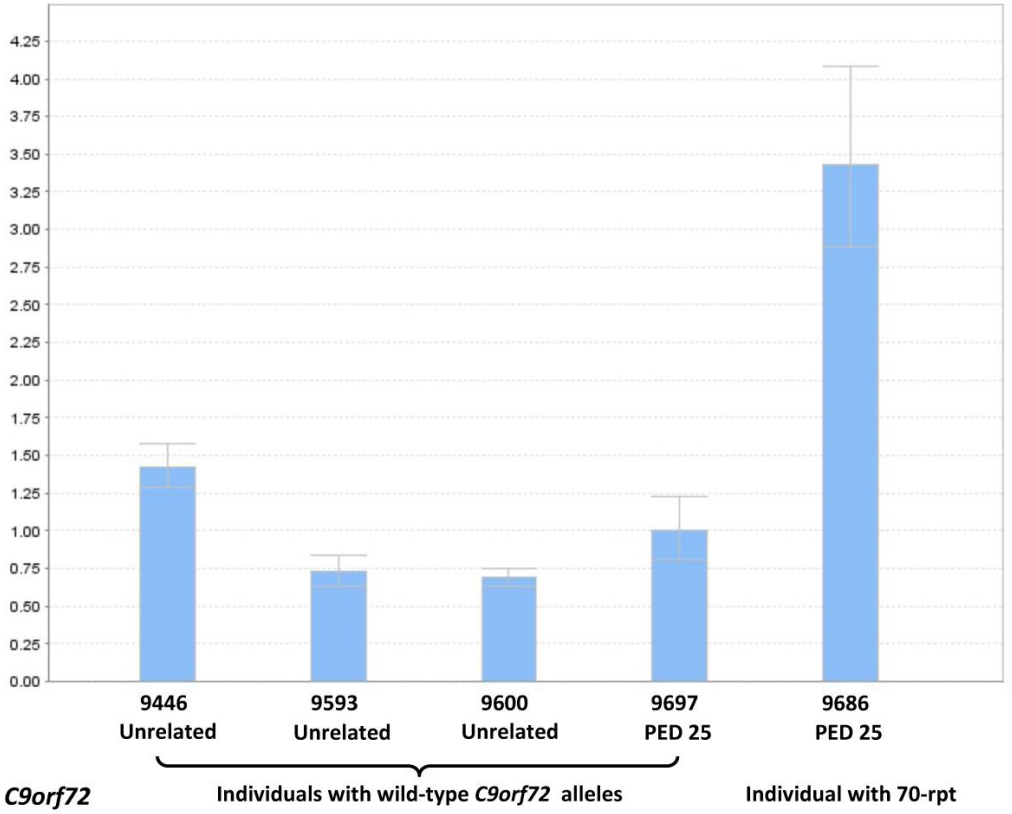


Figure S4. Relative quantification of *C9orf72* mRNA in blood from unrelated normal controls and the individual with the small expansion

The individual with the small expansion #9686 (2/~70) showed increased *C9orf72* gene expression compared to three unrelated normal controls: #9446 (2/22), #9593 (2/8) and #9600 (2/2), as well as PED25 family member #9697 with normal alleles. Bars represent the standard error of the triplicate reactions.

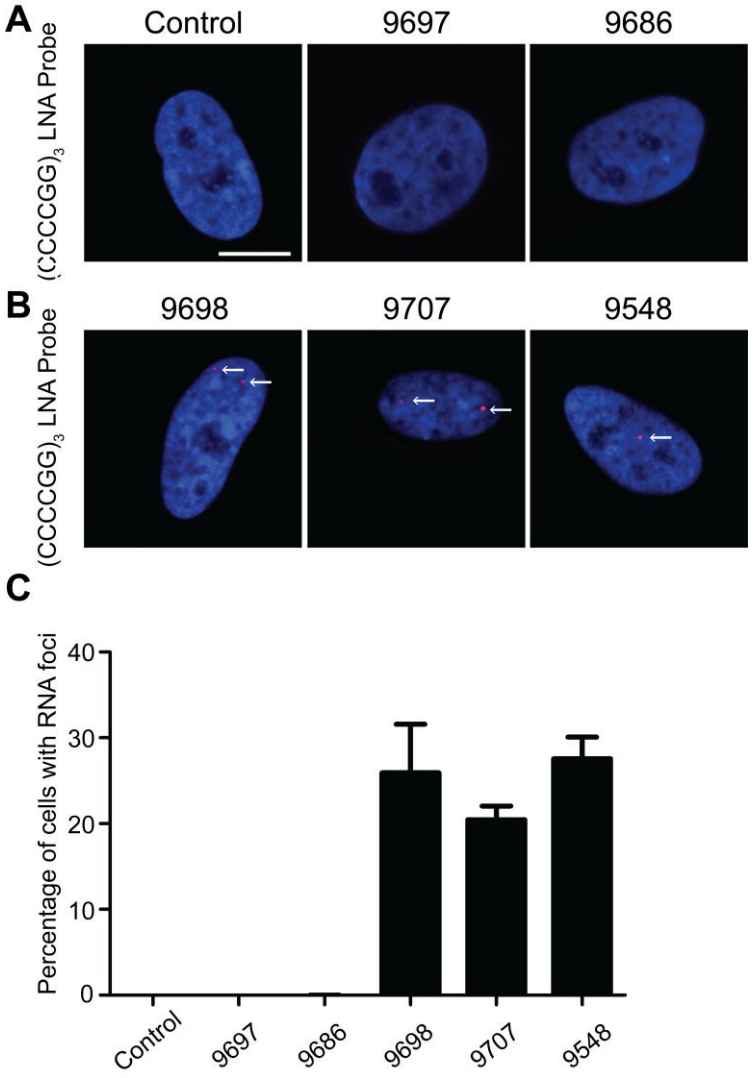


Figure S5. RNA foci were detected in fibroblasts from individuals with large expansions but not from the individual with the 70-repeat allele or unrelated control

RNA FISH using a probe complementary to the *C9orf72*-repeat expansion on fibroblasts from the control (#9697) or the individual with 70-repeat allele (#9686) did not detect RNA foci (**A**; scale bar = 10μm). However, RNA foci were detected in fibroblasts from subjects with large expansions: #9698, #9707 and #9548 (**B**, white arrows). Quantification of the number of RNA foci-positive cells in repeat expansion carriers revealed approximately 20-27% of cells to be positive for RNA foci (**C**). N = 3 experiments per fibroblast line, except 9686 (n = 2). Data are mean ± SEM. RNA FISH was based on previously published protocols [6; 7]. Briefly, fibroblasts plated on 13mm glass coverslips were washed with RNase-free PBS (Ambion) before fixation with 4% paraformaldehyde for 15 minutes at ambient temperature. Cells were permeabilized with PBS containing 0.2% (w/v) Triton X-100 for 10 minutes and washed once with 2 X saline sodium citrate (SSC) (Sigma) buffer. For prehybridization, cells were incubated with 2 X SSC containing 50% (v/v) formamide for 30 minutes at 60°C, followed by incubation with hybridization solution for 30 minutes at 60°C (50% formamide, 2 X SSC, 10% dextran, 0.2% bovine serum albumin, 2mM vanadyl ribonucleoside complex, 1mg/ml tRNA, 1mg/ml single stranded DNA from salmon sperm). A locked nucleic acid (LNA) probe, recognizing the sense strand (CCCCGG3) of the repeat expansion and with a 5'-TYE 563 fluorescent modification (Lagier-Tourenne et al., 2013; Exiqon catalogue number 607323), was denatured for 5 minutes at 85°C before addition to the hybridization solution to create a working concentration of 40nM. Cells were then incubated with probe for 2 hours at 60°C, and protected from light. This

was followed by three 20 minutes washes with 2 X SSC containing 50% formamide (v/v) at 60°C with shaking, and finally three 10 minute washes with 2 X SSC, with shaking. Coverslips were mounted with Prolong gold containing DAPI (Life Technologies). To quantify the number of cells expressing RNA foci, 10 random images were captured at 63x magnification per coverslip using a Leica DMI 6000 microscope with Volocity software (PerkinElmer). The number of RNA foci-positive cells was expressed as a percentage of total cells.

Table S1. Short tandem repeat (STR) markers and single nucleotide polymorphisms (SNPs) for subjects with small expansions or repeats in the long wild-type range

		1 (FTD)		2 (AD)		3 (FTD/MND)	
Marker	**Mb**	a1	a2	a1	a2	a1	a2
D9S171	24.53	175	183	185	167	169	185
D9S1679	24.78	135	131	135	131	131	135
D9S259	26.02	288	292	288	294	288	288
D9S2154	26.17	146	150	150	158	150	150
*rs4879515	27.48	T	C	T	C	T	C
*rs3849942	27.54	A	G	A	G	A	G
**C9orf72*	27.57	EXP	2	EXP	2	EXP	2
D9S161	27.63	129	131	117	129	117	127
D9S319	29.55	171	174	171	171	163	171

		4 (CON)		5 (CON)		6 (MND)		7 (AD)		8 (CON)		9 (CON)		10 (MND)		11 (LBD)		12 (MND)		13 (AD)		14 (CON)	
Marker	**Mb**	a1	a2	a1	a2	a1	a2	a1	a2	a1	a2	a1	a1	a1	a2	a1	a2	a1	a2	a1	a2	a1	a2
D9S171	24.5	167	175	167	175	175	181	167	183	167	175	169	175	169	179	167	183	183	185	169	175	175	181
D9S1679	24.8	131	135	135	135	135	131	135	137	131	135	131	135	131	135	133	135	131	133	131	137	131	131
D9S259	26.0	288	294	280	280	288	280	280	292	280	292	288	292	280	292	288	292	288	280	292	292	280	288
D9S2154	26.2	158	158	150	154	146	150	154	154	146	150	150	154	150	150	134	150	146	154	154	158	134	150
*rs4879515	27.5	T	C	T	T	T	T	T	C	T	C	T	T	T	C	T	T	T	T	T	T	T	C
*rs3849942	27.5	A	G	A	G	A	G	A	G	A	G	A	A	A	G	A	A	A	A	A	G	A	G
**C9orf72*	27.6	21	2	21	5	21	2	21	2	22	2	23	8	23	2	27	20	27	10	27	5	27	2
D9S161	27.6	119	127	125	127	129	131	117	131	117	129	127	129	119	127	117	127	127	127	127	131	117	117
D9S319	29.5	171	175	167	167	163	167	171	174	167	175	159	167	167	171	167	171	167	171	167	171	171	174

Subjects are diagnosed with frontotemporal dementia (FTD), Alzheimer's disease (AD), FTD with motor neuron disease (FTD/MND), MND, or Lewy Body Dementia (LBD); the remaining subjects are unaffected (CON). The *C9orf72* row displays the number of repeats on allele 1 (a1) and allele 2 (a2). Repeat expansions are indicated with 'EXP'. A Southern blot showing the small repeat expansion for subject #1 and #2 (subject #3 has been published elsewhere [8]); and two representative samples with long wild-type alleles are provided in Figure S3. All samples share a small region that is thought to represent the risk-haplotype (denoted by the asterisks).

Supplemental References

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