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Deep Intronic *FGF14* GAA Repeat Expansion in Late-Onset Cerebellar Ataxia

A full list of authors and affiliations appears at the end of the article.

Abstract

BACKGROUND—The late-onset cerebellar ataxias (LOCAS) have largely resisted molecular diagnosis.

METHODS—We sequenced the genomes of six persons with autosomal dominant LOCA who were members of three French Canadian families and identified a candidate pathogenic repeat expansion. We then tested for association between the repeat expansion and disease in two independent case–control series — one French Canadian (66 patients and 209 controls) and the other German (228 patients and 199 controls). We also genotyped the repeat in 20 Australian and 31 Indian index patients. We assayed gene and protein expression in two postmortem cerebellum specimens and two induced pluripotent stem-cell (iPSC)–derived motor-neuron cell lines.

RESULTS—In the six French Canadian patients, we identified a GAA repeat expansion deep in the first intron of *FGF14*, which encodes fibroblast growth factor 14. Cosegregation of the repeat expansion with disease in the families supported a pathogenic threshold of at least 250 GAA repeats ($[GAA]_{250}$). There was significant association between *FGF14* $(GAA)_{250}$ expansions and LOCA in the French Canadian series (odds ratio, 105.60; 95% confidence interval [CI], 31.09 to 334.20; P<0.001) and in the German series (odds ratio, 8.76; 95% CI, 3.45 to 20.84; P<0.001). The repeat expansion was present in 61%, 18%, 15%, and 10% of French Canadian, German, Australian, and Indian index patients, respectively. In total, we identified 128 patients with LOCA who carried an *FGF14* $(GAA)_{250}$ expansion. Postmortem cerebellum specimens and iPSC-derived motor neurons from patients showed reduced expression of *FGF14* RNA and protein.

CONCLUSIONS—A dominantly inherited deep intronic GAA repeat expansion in *FGF14* was found to be associated with LOCA. (Funded by Fondation Groupe Monaco and others.)

LATE-ONSET CEREBELLAR ATAXIAS (LOCAs) are a heterogeneous group of neurodegenerative disorders manifesting as a progressive cerebellar syndrome that develops after 30 years of age.[1] The prevalence of LOCA is approximately 1 to 3 per 100,000 population,[2] and molecular testing yields negative results in almost 75% of patients with LOCA.[3] This is explained in part by the limitations of standard next-generation sequencing analysis for the identification of certain sequence variations, such as tandem repeat expansions.[4] We used bioinformatics tools and long-read sequencing to search for novel pathogenic repeat expansions in patients with LOCA

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METHODS

ENROLLMENT, IMAGING, AND NEUROPATHOLOGICAL ASSESSMENT

To be eligible for inclusion in the study, patients needed to have progressive ataxia with onset at or after the age of 30 years; no clinical features suggestive of multiple system atrophy, cerebellar subtype (MSA-c); exclusion of acquired disease; and, as appropriate, negative results on testing with an ataxia gene panel and on screening for repeat expansions that cause common spinocerebellar ataxias, fragile X–associated tremor–ataxia syndrome, Friedreich's ataxia, and CANVAS (cerebellar ataxia, neuropathy, and vestibular areflexia syndrome).

Two persons with autosomal dominant LOCA from each of three large French Canadian families (Families I, II, and III) made up the discovery cohort (Fig. 1A). The six patients had LOCA for which an underlying genetic cause had not yet been identified (i.e., unsolved LOCA). We also enrolled independent validation cohorts of 66 French Canadian, 228 German, 20 Australian, and 31 Indian index patients. The control cohorts consisted of 209 French Canadian and 199 German persons who were neurologically healthy or who were reported not to have ataxia.

Aside from 14 patients whose ancestry was determined by analysis of next-generation sequencing data, participants reported their own race and ethnic group. Information on the representativeness of the study participants is provided in Table S1 in the Supplementary Appendix, available with the full text of this article at NEJM.org. Brain images were obtained by magnetic resonance imaging (MRI), and comprehensive neuropathological examinations were conducted on the brains of two deceased French Canadian patients. Details of the staining and immunohistochemical analysis are provided in the Supplementary Appendix.

We obtained written informed consent from all the participants in the study, and all local institutional review boards approved the study. The authors vouch for the accuracy and completeness of the data in this report.

GENETIC STUDIES

We sequenced the genomes[5,6] of the six French Canadian patients from the discovery cohort and searched for novel pathogenic repeat expansions with the use of ExpansionHunter Denovo software, version 0.6.2.[7] Participants in the 1000 Genome Project and the Vanderbilt Atrial Fibrillation Registry served as controls.

The *FGF14* repeat locus was amplified by long-range polymerase chain reaction (PCR). The number of repeat units was estimated by means of agarose-gel electrophoresis. The motif of the repeat locus in patients and controls who had large amplification products on long-range PCR was analyzed by targeted long-read nanopore sequencing. For patients with insufficient DNA available for nanopore sequencing, we used repeat-primed PCR to ascertain the presence of a GAA repeat expansion. Targeted long-read nanopore sequencing was performed on specimens from 104 patients (55 French Canadian, 44 German, and 5 Australian) and 18 controls.

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ASSOCIATION STUDIES

We tested for association between LOCA and *FGF14* GAA expansions of 250 or more repeats ([GAA] $_{250}$), as measured by agarose-gel electrophoresis of PCR-amplification products, in two independent case–control series — one French Canadian (66 patients and 209 controls) and the other German (228 patients and 199 controls). We used 250 or more repeat units for association studies; this choice was guided by the results of the segregation study within the discovery cohort, which showed that the smallest expansion among symptomatic persons was $(GAA)_{250}$. The study workflow is summarized in Figure S1.

FUNCTIONAL ANALYSES

Postmortem Cerebellar Tissue—Postmortem cerebellar tissue was obtained from two patients of Spanish origin who carried an *FGF14* GAA repeat expansion that had been uncovered after screening of postmortem brain specimens from 15 patients of European descent with unsolved LOCA and 7 controls of European descent without ataxia. The brain specimens were obtained through the Queen Square Brain Bank for Neurological Disorders, London, and the Neurological Tissue Brain Bank of the Hospital Clínic–IDIBAPS (Instituto de Investigaciones Biomédicas August Pi i Sunyer) Biobanc, Barcelona (Table S2).

Induced Pluripotent Stem Cell–Derived Motor Neurons—We generated induced pluripotent stem-cell (iPSC) lines — one from a patient carrying a $(GAA)_{383}$ expansion, one from a patient homozygous for $(GAA)_{300}$ expansions, and two from unaffected persons of European descent — and differentiated them into motor neurons using an established protocol.[8] Although neither of the affected persons had clinical features consistent with motor-neuron disease, the challenges of establishing mature Purkinje cell lines in culture[9] led us to generate iPSC-derived motor neurons. Details of the methods used to assay gene and protein expression are provided in the Supplementary Appendix.

STATISTICAL ANALYSIS

A Fisher's exact test was used for tests of association. No method to adjust for multiplicity of inferences was prespecified. The widths of the confidence intervals have not been adjusted for multiplicity, and therefore the confidence intervals should not be used for hypothesis testing.

RESULTS

IDENTIFICATION OF A GAA REPEAT EXPANSION IN *FGF14*

We selected six patients with unsolved autosomal dominant LOCA from the three large French Canadian families (Fig. 1A) for genome sequencing because they shared a similar phenotype that included episodic features and downbeat nystagmus. Before this study, linkage analysis in Family I had not identified candidate loci as a result of the likely presymptomatic status of some of the younger members. Initial analysis[6] of the genome sequences revealed no rare pathogenic variants segregating across all three pedigrees.

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We next used ExpansionHunter Denovo[7] and identified a putative heterozygous GAA repeat expansion shared among all six patients at position chr13:102,813,925–102,814,074 (GRCh37) (Fig. 1B and 1C, Fig. S2, and Table S5). This repeat expansion is located in intron 1 of *FGF14*, which is included in pre-mRNA transcript 2 and not in pre-mRNA transcript 1 (Fig. 1B). Exonic point and frameshift variants in *FGF14* have previously been shown to cause autosomal dominant spinocerebellar ataxia 27 (SCA27).[10,11] Long-range PCR and repeat-primed PCR showed GAA repeat expansions in the six patients and in 15 affected relatives (Fig. 1D, 1E, and 1F and Fig. S3). Sanger sequencing confirmed the repeat motif to be GAA. The smallest expansion in any affected family member was $(GAA)_{250}$.

ANALYSIS OF THE *FGF14* REPEAT LOCUS IN CONTROLS

We studied the allelic distribution of the repeat locus by means of long-range PCR in 408 controls (209 French Canadian and 199 German) and observed wide variation in repeat sizes (Fig. 2A and Fig. S4). Two thirds of alleles (544 of 816) carried 25 or fewer repeat units. Of the 816 control chromosomes, 11 carried an expansion of at least 250 triplet repeats, as assessed by PCR. Targeted long-read sequencing of these 11 alleles showed that 8 consisted of $(GAA)_n$ repeats, two of $(GAAGGA)_n$ repeats, and one of $[(GAA)_4(GCA)_1]_n$ repeats. We established that the allele frequency of $(GAA)_{250–300}$ expansions was 0.98% among controls and that none of the 816 control chromosomes surveyed carried $(GAA)_{>300}$ expansions (Figs. S5 and S6). We were unable to clinically assess all controls carrying an expansion to determine whether they had ataxia.

FGF14 GAA REPEAT EXPANSION IN INDEPENDENT COHORTS OF PATIENTS WITH LOCA

We next screened four independent cohorts of patients with unsolved LOCA from Quebec, Germany, Western Australia, and India for the presence of the *FGF14* repeat expansion (Fig. 2B, 2C, and 2D and Figs. S4, S10, S11, and S12). These cohorts consisted of 66 French Canadian index patients (excluding members of Families I, II, and III), 228 German index patients, 20 Australian index patients, and 31 Indian index patients.

Among the 66 French Canadian patients, 40 (61%) carried a $(GAA)_{250}$ expansion, as compared with 3 of 209 French Canadian controls (1%) (odds ratio, 105.60; 95% confidence interval [CI], 31.09 to 334.20; $P<0.001$ by Fisher's exact test of association). An *FGF14* $(GAA)_{250}$ expansion was also present in 12 affected relatives from nine families that included an index patient. The diagnostic yield of the repeat expansion in the French Canadian index cohort was 75% (24 of 32) among patients with early episodic features, 93% (26 of 28) among patients with downbeat nystagmus, and 94% (17 of 18) among patients with a combination of early episodic features and downbeat nystagmus.

Of the 228 German patients, 42 (18%) carried a $(GAA)_{250}$ expansion, as compared with 5 of 199 (3%) German controls (odds ratio, 8.76; 95% CI, 3.45 to 20.84; $P<0.001$ by Fisher's exact test of association). The expansion was also present in 7 affected relatives from six German families that included an index patient. Furthermore, 3 of 20 Australian index patients (15%) and 3 of 31 Indian index patients (10%) carried a repeat expansion in *FGF14* (Fig. 2D). We thus found a total of 128 patients (73 French Canadian, 49 German, 3 Australian, and 3 Indian) with an *FGF14* $(GAA)_{250}$ expansion and, accordingly, GAA-

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FGF14–related ataxia. Four of the patients were homozygous or compound heterozygous for $(GAA)_{250}$ expansions, and 2 were compound heterozygous for a $(GAA)_{250}$ expansion and a $(GAAGGA)_{125}$ expansion. Our data suggest that $(GAA)_{250–300}$ expansions are likely to be pathogenic, albeit with reduced penetrance, and that $(GAA)_{>300}$ expansions are fully penetrant.

Two German and two Australian patients carried a $(GAAGGA)_n$ expansion (Figs. S13 and S14). The absence of segregation of this hexanucleotide repeat expansion with disease in affected family members from the two Australian families suggests that non-GAA expansions at the *FGF14* locus are not associated with LOCA.

All but two patients with GAA-*FGF14*–related ataxia in the French Canadian, German, and Australian cohorts were of European ancestry (see the Supplementary Results section and Fig. S15). The two non-European patients were from northeastern Turkey. All three Indian patients were of South Asian ancestry. Future studies of the prevalence of GAA-*FGF14*–related ataxia in other populations will be necessary to fully define its epidemiologic characteristics.

The high proportion of French Canadian patients carrying the *FGF14* expansion may correspond to a founder effect in this population, in which such effects are known to be common.[12] In a finding consistent with this observation, a candidate 693-kb haplotype delimited by the polymorphisms rs12856547 and rs34644481 was shared by the 10 patients belonging to Family I and, at least in part, by the 4 other French Canadian patients for whom genome sequences were available (see the Supplementary Results section and Fig. S18). The fact that GAA-*FGF14*–related ataxia was found in patients of Turkish and Indian descent suggests that this repeat expansion may arise on multiple haplotype backgrounds. Future studies will be necessary to confirm whether this candidate disease haplotype is found in non–French Canadian patients.

GERMLINE INSTABILITY OF THE GAA REPEAT EXPANSION

The transmission of expanded $(GAA)_{250}$ alleles invariably resulted in expansion in the female germline and contraction in the male germline across 30 meiotic events that we studied (Fig. 2E). Four meiotic events in an affected father carrying $(GAA)_{300/716}$ expansions (Patient II.1, Fig. 1A) resulted in the inheritance of subpathogenic alleles (216, 225, 233, and 233 repeats) in the asymptomatic offspring. Reduced male transmission of the disease was further supported by the observation that only 20 of 66 dominantly inherited cases (30%) were paternally inherited.

CLINICAL AND RADIOLOGIC FEATURES

Table 1 summarizes the main clinical features of 122 of the 128 patients with confirmed GAA-*FGF14*–related ataxia. Overall, the ataxia was reported as episodic at onset in 46% of the patients. Episodes consisted of variable combinations of diplopia, vertigo, dysarthria, and appendicular and gait ataxia that lasted from minutes to days, with a daily to monthly occurrence. The most commonly reported triggers were alcohol intake and exercise. Two of the 47 French Canadian patients (4%) who presented with episodic disease initially received a misdiagnosis of acute peripheral vestibular syndrome. The mean (±SD) age at the

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onset of episodic symptoms and at the onset of progressive ataxia was 55±13 years (range, 30 to 87) and 59±11 years (range, 30 to 88), respectively. We observed a weak inverse correlation between the age at onset and the size of the repeat expansion (120 patients; Pearson correlation coefficient, −0.31; $R^2 = 0.10$) (see the Supplementary Results section and Fig. S19).

A substantial percentage of patients eventually used a walking aid (58%; 65 of 112), including the use of a wheelchair in 13% of patients (15 of 112). Downbeat nystagmus was observed in 42% of patients; this finding was especially prominent during episodes of ataxia. Vestibular areflexia was documented in 5 patients. Spasticity was found on examination in 8% of patients (9 of 120). Nerve conduction studies in 28 patients with suspected polyneuropathy on clinical examination revealed at most a mild peripheral axonal sensory neuropathy in 7 patients, which suggests that polyneuropathy is not a core feature of GAA-*FGF14*–related ataxia. MRI of the head showed variable degrees of cerebellar atrophy in 74% of patients (67 of 91) (Fig. 3A). Acetazolamide was mildly beneficial in 9 of the 23 patients to whom it was given. In comparison, treatment with 4-aminopyridine resulted in a marked to moderate reduction in the frequency or severity of ataxic symptoms in 7 of 8 patients.

NEUROPATHOLOGICAL EXAMINATION

Neuropathological examination of the brain from two of the French Canadian patients revealed cerebellar atrophy that was more prominent in the vermis than in the hemispheres. Cerebellar atrophy was present in the form of widespread depletion of Purkinje cells, gliosis in the molecular layer, and overall mild cell loss in the granule-cell layer (Fig. 3B through 3J). Dentate nuclei showed no substantial atrophy, and the cerebellar white matter showed substantial pallor only in the subcortical regions of the vermis. No apparent atrophy in any region was seen on macroscopic and microscopic examination of brain stem and spinal cord. Immunostaining for p62 showed no evidence of pathological intranuclear or cytoplasmic inclusions in the cerebellum (see the Supplementary Results section).

MECHANISTIC STUDIES

We used postmortem cerebellum specimens and iPSC-derived motor neurons to investigate the mechanisms by which the GAA repeat expansion leads to pathogenicity. Two patients of Spanish origin who carried a GAA repeat expansion — Patient 1, carrying a $(GAA)_{300}$ expansion, and Patient 2, carrying a $(GAA)_{350}$ expansion — were found after screening of postmortem brains from 15 persons with unsolved LOCA. Both patients had late-onset episodic ataxia (ages at onset, 78 and 71 years) and downbeat nystagmus. Quantitative PCR showed 19.1% lower *FGF14* transcript 2 expression among the patients than among controls (Fig. 4A and 4B). The observation of a 22.0% lower total *FGF14* expression among the patients provided further evidence for decreased transcript 2 expression, given that it normally has higher expression than transcript 1 in the cerebellum.[13] Immunoblotting showed 54.6% lower FGF14 levels in patient cerebellum than in control cerebellum across triplicate experiments (Fig. 4C and 4D).

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In the iPSC-derived motor neurons, levels of transcript 1 could not be reliably assessed by quantitative PCR because of the low level of expression. The level of transcript 2 expression was 84.3% lower in one patient and 94.2% lower in the other patient than in control cells (Fig. 4E). Consistent results were obtained in an assay of total *FGF14* expression (Fig. S20). Western blot analysis showed FGF14 protein levels that were 54.3% lower in the first patient and 75.0% lower in the second patient than in controls (Fig. 4F and 4G). The possibility of codominance of the *FGF14* repeat expansion was suggested by the consistently lower levels of transcript 2 expression and FGF14 protein in the patient who was homozygous for *FGF14* GAA expansions, as compared with the other patient, who was heterozygous. Taken together, data obtained from examinations of postmortem cerebellum and induced motor neurons suggest that the intronic GAA expansion in *FGF14* leads to haploinsufficiency. The possibility that additional mechanisms contribute to pathogenicity of the GAA repeat expansion will require further exploration.

DISCUSSION

We identified a novel GAA repeat expansion in the first intron of *FGF14* in persons with LOCA. The *FGF14* $(GAA)_{250}$ expansion is pathogenic according to criteria of the American College of Medical Genetics and Genomics[14] because it is significantly more prevalent among affected persons than among controls (odds ratio >5.0, with a confidence interval around the estimate of odds ratio that does not include 1.0), it has a damaging effect on the gene product, and it cosegregates with disease in multiple affected family members. According to the ClinGen framework,[15] we have provided strong support for pathogenicity of the *FGF14* GAA repeat expansion. Our results support incomplete penetrance of $(GAA)_{250–300}$ expansions and full penetrance of $(GAA)_{>300}$ expansions. As with other repeat expansion disorders, we expect that the study of larger cohorts will refine diagnostic thresholds.

Despite its prevalence in LOCA, the *FGF14* GAA repeat expansion had, until now, resisted identification. Among contributing factors are the high degree of polymorphism of this repeat locus in the general population, the reduced male transmission of the disease, and the late onset of disease.

Several lines of evidence suggest that the *FGF14* GAA repeat is highly unstable. First, the number of *FGF14* GAA repeats varies from 8 to 300 in unaffected persons. In comparison, the *FXN* repeat length ranges from 5 to 33 repeats among unaffected persons.[16] Second, although a common disease haplotype was shared by the 14 French Canadian patients for whom haplotype analysis was performed in our study, the observation of the repeat expansion in Turkish and Indian patients suggests that it may arise on distinct haplotype backgrounds. Third, the relatively high incidence of sporadic GAA-*FGF14*–related ataxia may be a consequence of frequent stochastic expansion of the GAA repeat in the general population as a result of instability of intermediate alleles during parent–offspring transmission. Finally, large contractions of the GAA expansion in the male germline can lead to transmission of subpathogenic alleles to offspring and generation skipping of the disease. Contraction of repeat expansions during paternal meiosis has also been documented

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in Friedreich's ataxia[17] and other disorders caused by noncoding trinucleotide repeat expansions.[18]

Preliminary investigations of patient-derived postmortem cerebellum and induced motor neurons suggest that the intronic GAA expansion leads to loss of function by interfering with *FGF14* transcription. This mechanism would be consistent with reports that SCA27 is caused by haploinsufficiency of *FGF14*,[10] as well as with the high intolerance of *FGF14* to loss of function (probability of being loss-of-function intolerant, 0.91; ratio of observed number to expected number of loss-of-function variants, 0.08; 90% CI, 0.03 to 0.39).[19] However, the elucidation of the mechanism through which the GAA repeat expansion causes *FGF14* transcriptional deficiency will require further study. Perhaps it involves epigenetic silencing, as has been shown with *FXN* in Friedreich's ataxia.[20]

Loss of *FGF14* function causes ataxia in *Fgf14*-knockout mice.[21] *FGF14* is expressed throughout the central nervous system, most abundantly in cerebellar granule and Purkinje cells.[22] It is involved in spontaneous rhythmic firing of Purkinje cells by regulating and promoting localization of voltage-gated sodium channels at the axon initial segment.[23–25] Impairment of ion-channel kinetics, as previously shown with *Fgf14* knockdown in mouse Purkinje cells,[26] is consistent with the frequent episodic presentation in GAA-*FGF14*–related ataxia. These observations suggest that GAA-*FGF14*–related ataxia may be a type of channelopathy.

As compared with GAA-*FGF14*–related ataxia, SCA27 typically manifests at an earlier age (mean, 23.7 years), is frequently associated with early-onset postural tremor (96%) and neuropsychiatric manifestations (56%), and is less commonly associated with cerebellar atrophy (20%).[27] Although our data suggest that most patients have disease onset in the sixth decade of life, we cannot rule out larger expansions being associated with ataxia occurring before 30 years of age. When the possibility of screening affected persons for the *FGF14* GAA repeat expansion is being considered, the fact that isolated downbeat nystagmus is commonly observed early in the disease course should be noted. Consistent with this observation is the association between a genetic marker in *FGF14* (rs72665334, which is close to the repeat locus) and idiopathic downbeat nystagmus in a previous genomewide association study.[28] The observation of vestibular areflexia in a subgroup of patients suggests partial phenotypic overlap between GAA-*FGF14*–related ataxia and *RFC1*-related ataxia. GAA-*FGF14*–related ataxia can be distinguished from MSA-c, in which the age at onset is similar,[29] on the basis of its frequent positive family history, slower progression, and, when present, isolated cerebellar atrophy on MRI.

We identified a dominant GAA repeat expansion in the first intron of *FGF14* in persons with unsolved LOCA. Our findings also show how late-onset disorders can be associated with individual alleles of strong genetic effect. Our study underscores the importance of identifying noncoding repeat expansions, because they probably account for some of the missing heritability in unsolved neurodegenerative disorders.[18]

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Supplementary Material

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Authors

David Pellerin, M.D.,
Matt C. Danzi, Ph.D.,
Carlo Wilke, M.D.,
Mathilde Renaud, M.D., Ph.D.,
Sarah Fazal, Ph.D.,
Marie-Josée Dicaire, B.Sc.,
Carolin K. Scriba, B.Sc.,
Catherine Ashton, M.B., B.S.,
Christopher Yanick, B.S.,
Danique Beijer, Ph.D.,
Adriana Rebelo, Ph.D.,
Clarissa Rocca, M.Sc.,
Zane Jaunmuktane, M.D.,
Joshua A. Sonnen, M.D.,
Roxanne Larivière, Ph.D.,
David Genís, M.D.,
Laura Molina Porcel, M.D., Ph.D.,
Karine Choquet, Ph.D.,
Rawan Sakalla, B.Sc.,
Sylvie Provost, M.Sc.,
Rebecca Robertson, Ph.D.,
Xavier Allard-Chamard, M.Sc.,
Martine Tétreault, Ph.D.,
Sarah J. Reiling, Ph.D.,
Sara Nagy, M.D.,
Vikas Nishadham, M.B., B.S.,
Meera Purushottam, Ph.D.,
Seena Vengalil, M.D.,
Mainak Bardhan, M.B., B.S.,
Atchayaram Nalini, M.D., Ph.D.,
Zhongbo Chen, B.M., B.Ch.,
Jean Mathieu, M.D.,
Rami Massie, M.D.,
Colin H. Chalk, M.D.,
Anne-Louise Lafontaine, M.D.,

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François Evoy, M.D.,
Marie-France Rioux, M.D.,
Jiannis Ragoussis, Ph.D.,
Kym M. Boycott, M.D., Ph.D.,
Marie-Pierre Dubé, Ph.D.,
Antoine Duquette, M.D.,
Henry Houlden, M.D., Ph.D.,
Gianina Ravenscroft, Ph.D.,
Nigel G. Laing, Ph.D.,
Phillipa J. Lamont, M.B., B.S., Ph.D.,
Mario A. Saporta, M.D., Ph.D.,
Rebecca Schüle, M.D.,
Ludger Schöls, M.D.,
Roberta La Piana, M.D., Ph.D.,
Matthis Synofzik, M.D.,
Stephan Zuchner, M.D., Ph.D.,
Bernard Brais, M.D., C.M., Ph.D.

Affiliations

Departments of Neurology and Neurosurgery (D.P., M.-J.D., J.A.S., R.L., R. Sakalla, R.R., X.A.-C., R.M., C.H.C., A.-L.L., R.L.P., B.B.) and Pathology (J.A.S.), Montreal Neurological Hospital and Institute, McGill Genome Centre, Department of Human Genetics (S.J.R., J.R.), and the Departments of Diagnostic Radiology (R.L.P.) and Human Genetics (K.C., R.R., X.A.-C., B.B.), McGill University, Montreal Heart Institute (S.P., M.-P.D.), the Departments of Neurosciences (M.T., A.D.) and Medicine (M.P.D.), Faculty of Medicine, Université de Montréal, Université de Montréal Beaulieu-Saucier Pharmacogenomics Center (S.P.), Centre de Recherche du Centre Hospitalier de l'Université de Montréal (M.T., A.D.), and Centre de Réadaptation Lucie-Bruneau (A.D., B.B.), Montreal, the Faculty of Medicine and Health Sciences, Sherbrooke University, Sherbrooke, QC (J.M., F.E., M.-F.R.), and Children's Hospital of Eastern Ontario Research Institute, University of Ottawa, Ottawa (K.M.B.) — all in Canada; the Department of Neuromuscular Disease, UCL Queen Square Institute of Neurology and the National Hospital for Neurology and Neurosurgery (D.P., C.R., S.N., H.H.), the Department of Clinical and Movement Neurosciences and Queen Square Brain Bank for Neurological Disorders (Z.J.) and the Department of Neurodegenerative Disease (Z.C.), UCL Queen Square Institute of Neurology, University College London, and the Division of Neuropathology, National Hospital for Neurology and Neurosurgery, University College London NHS Foundation Trust (Z.J.) — all in London; Dr. John T. Macdonald Foundation Department of Human Genetics and John P. Hussman Institute for Human Genomics (M.C.D., S.F., C.Y., D.B., A.R., S.Z.), and the Department of Neurology (C.Y., M.A.S.), University of Miami Miller School of Medicine, Miami; the Department of Neurodegenerative Diseases, Hertie Institute for Clinical Brain Research

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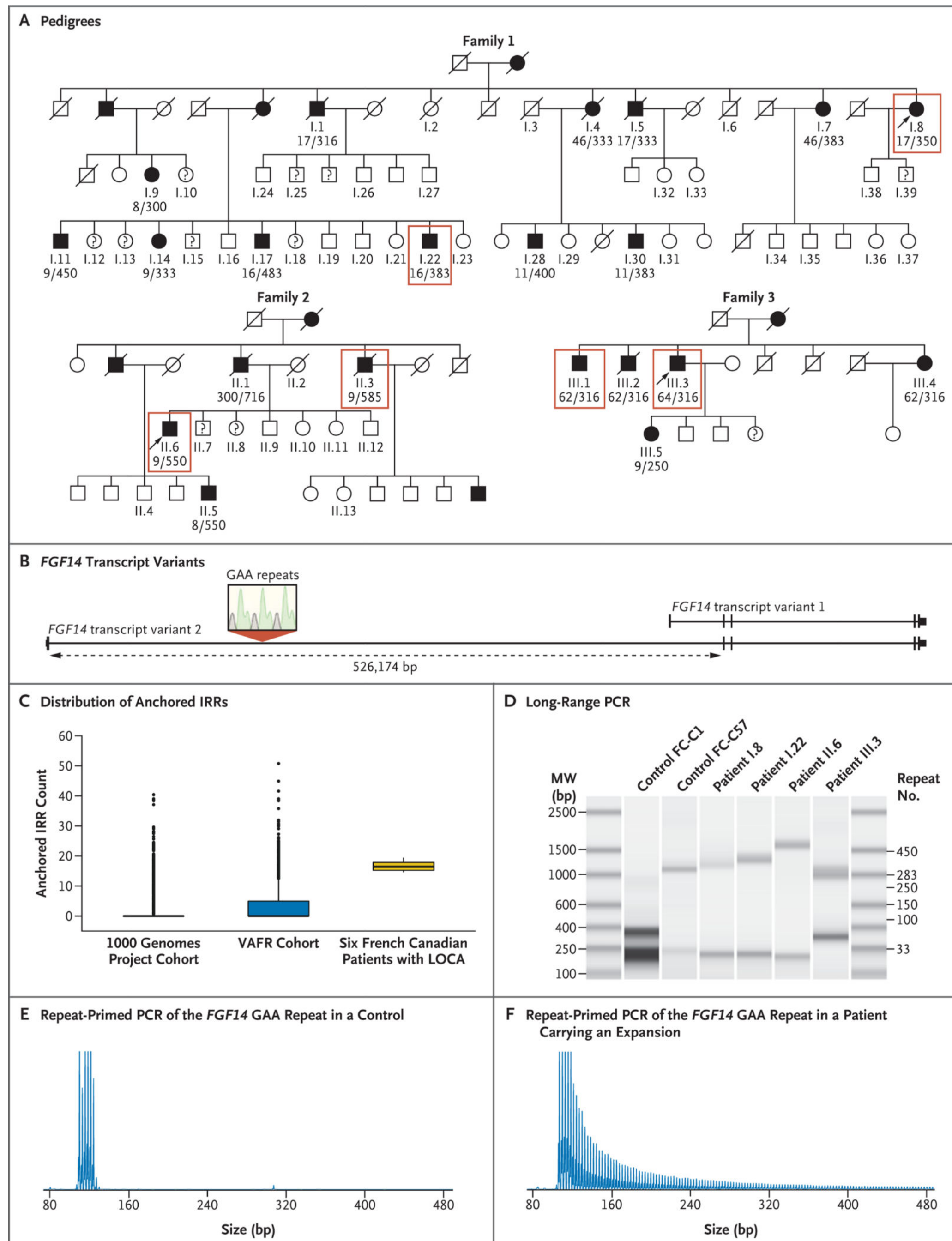

Figure 1. Identification of a Deep Intronic GAA Repeat Expansion in *FGF14* in Patients with Late-Onset Cerebellar Ataxia.

Panel A shows the pedigrees of three large unrelated French Canadian families with unsolved autosomal dominant late-onset cerebellar ataxia (LOCA). Numbered family members underwent genotyping for the GAA repeat expansion in *FGF14* by long-range polymerase chain reaction (PCR) and repeat-primed PCR. Allele sizes expressed as numbers of GAA repeats are provided for clinically affected persons only. Family members for whom whole-genome sequences were obtained are indicated by a red box. Squares represent

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male family members, and circles female family members. Solid black shapes indicate affected persons. Question marks indicate persons whose clinical status is uncertain. Slashed symbols indicate deceased persons. Probands are indicated by arrows. Pedigrees have been abbreviated and family members randomly rearranged to preserve privacy. **Panel B** is a diagram of *FGF14* transcript variants 1 (NM_004115.4) and 2 (NM_175929.3). The location of the $(GAA)_n$ repeat locus in the first intron of *FGF14* transcript variant 2 is indicated by the red arrowhead. **Panel C** shows the distribution of anchored in-repeat reads (IRRs) obtained with the use of ExpansionHunter Denovo for each of the 2504 samples from the 1000 Genomes Project control cohort, 1115 samples from the Vanderbilt Atrial Fibrillation Registry (VAFR) control cohort, and samples from 6 French Canadian patients with LOCA at the *FGF14* repeat locus (chr13:102,813,925–102,814,074; GRCh37). The mean (±SD) number of anchored IRRs at this locus was 16.68±1.90 (median, 16.40; range, 14.60 to 19.45) in the group of 6 patients with LOCA, which was higher than that in the 1000 Genomes Project data set (1.77±4.99; median, 0; range, 0 to 40.42; Cohen's d, 2.99; 95% confidence interval [CI], 2.19 to 3.80) and the VAFR cohort (4.38±7.73; median, 0; range, 0 to 50.78; Cohen's d, 1.59; 95% CI, 0.78 to 2.40). The widths of the confidence intervals have not been adjusted for multiplicity, and therefore the confidence intervals should not be used to reject or not reject effects. **Panel D** shows the results of long-range PCR indicating a large heterozygous expansion of the *FGF14* repeat locus. A representative image of PCR amplification products that were resolved with the use of the Agilent 4200 TapeStation automated electrophoresis system from two controls and four patients is shown. All samples were amplified and resolved during the same experiment. The four patients each carried one expanded product that was at least 900 bp in length, corresponding to 250 or more GAA repeats. The large amplification product of Control FC-C57 was found by long-read nanopore sequencing to be a GAAGGA hexanucleotide repeat expansion. MW denotes molecular weight. **Panels E and F** show the results of repeat-primed PCR of the *FGF14* GAA repeat unit. Panel E is a normal electropherogram in a control who was homozygous for $(GAA)_{11}$ alleles. Panel F is an electropherogram showing the characteristic sawtooth pattern in a patient with LOCA (Patient II.6) carrying an expanded $(GAA)_{550}$ allele.

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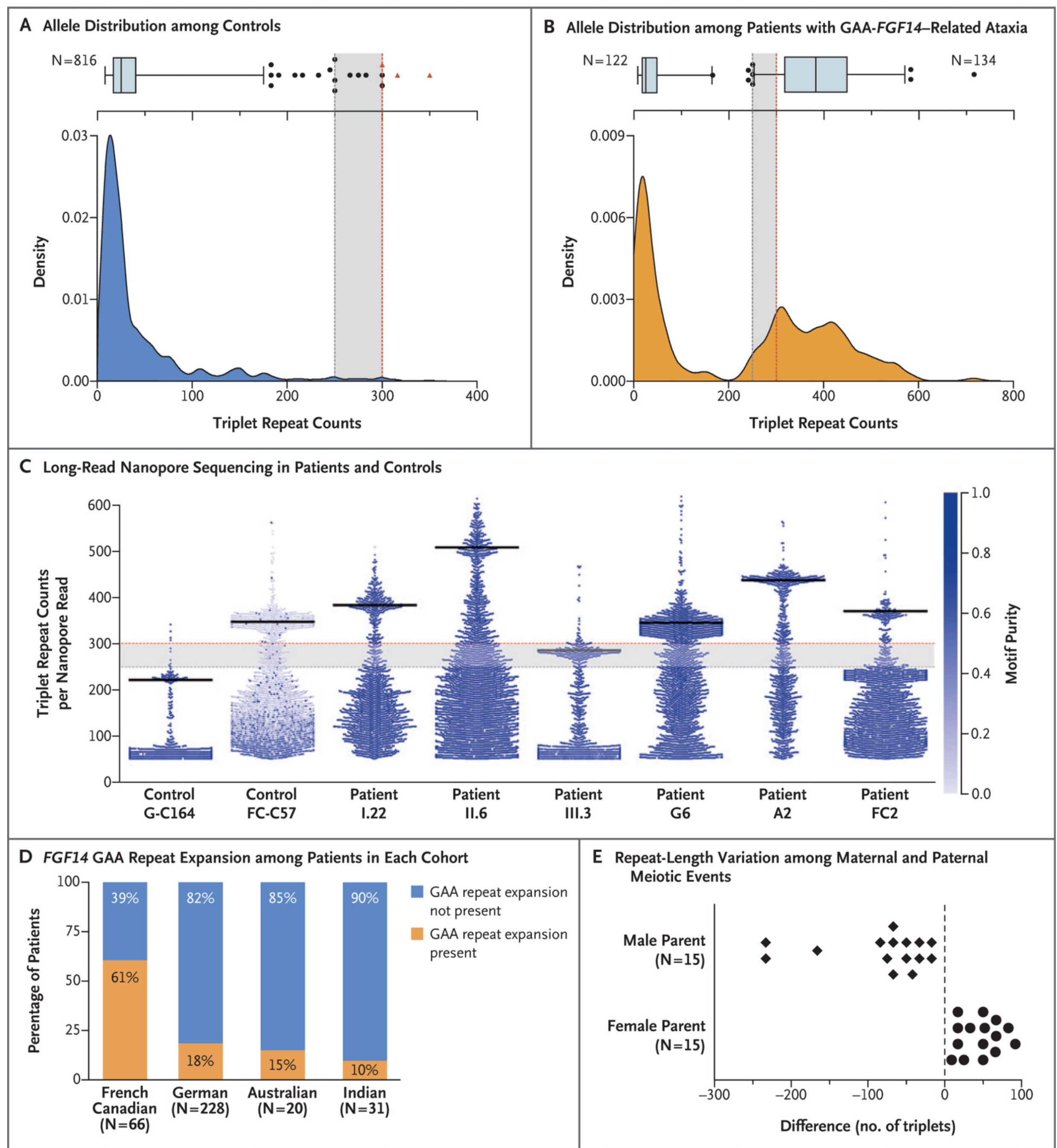

Figure 2. Allele Distribution and Long-Read Sequencing of the *FGF14* GAA Repeat Locus.
Panels A and B show the allele distribution of the *FGF14* repeat locus in 408 controls (816 chromosomes) (Panel A) and 128 patients with GAA-*FGF14*–related ataxia (122 normal and 134 expanded chromosomes) (Panel B). The repeat length was estimated by agarose-gel electrophoresis of PCR-amplification products. Among the patients with GAA-*FGF14*–related ataxia, 4 were homozygous or compound heterozygous for $(GAA)_{250}$ expansions, and 2 were compound heterozygous for a $(GAA)_{250}$ expansion and a $(GAAGGA)_{125}$ expansion. The smallest number of GAA repeats among controls and patients was 8.

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The density plots show allele-size frequencies, with higher densities indicating greater frequencies. The box-and-whisker plots show the allelic distribution in controls and patients. In Panel B, the box-and-whisker plots above the graph show the distribution of the normal alleles (left-hand plot) and expanded alleles (right-hand plot) in patients. The box indicates the 25th percentile (first quartile), the median, and the 75th percentile (third quartile), and the whiskers indicate the 2.5th and 97.5th percentiles. Outliers are represented by black dots. In controls, expanded alleles consisting of non-GAA repeats are represented by red triangles. The dashed gray lines and the shaded gray areas indicate the incompletely penetrant range of $(GAA)_{250–300}$, and the dashed red lines mark the threshold of $(GAA)_{300}$ repeat units, above which the alleles are fully penetrant. The allelic distributions in the different control and patient cohorts are shown in Figure S4. **Panel C** shows swarm plots of 3000 randomly sampled individual nanopore reads containing at least 50 repeat units for 2 controls and 6 patients. Control G-C164 carries a subpathogenic $(GAA)_{222}$ allele, and control FC-C57 carries a nonpathogenic $(GAAGGA)_{152}$ allele. Patients I.22, II.6, and III.3 (French Canadian discovery cohort) carry a $(GAA)_{388}$, $(GAA)_{508}$, and $(GAA)_{285}$ expansion, respectively. Patients G6 (German cohort) and A2 (Australian cohort) carry a $(GAA)_{345}$ and $(GAA)_{437}$ expansion, respectively. Patient FC2 (French Canadian cohort) carries a $(GAA)_{223}$ allele and a $(GAA)_{355}$ allele. Horizontal black bars indicate repeat size of the larger allele, as measured by nanopore sequencing. Expansion sizing by long-read nanopore sequencing and agarose-gel electrophoresis of PCR amplification products are highly similar (Pearson correlation coefficient, 0.96), as shown in Figure S8. The horizontal dashed gray line and the shaded gray area show the incompletely penetrant range of $(GAA)_{250–300}$, and the dashed red line marks the threshold of $(GAA)_{300}$ repeat units, above which the alleles are fully penetrant. The color of the data points is a function of the GAA repeat motif purity in each individual read, with dark blue indicating pure and lighter blue impure motif (a hue scale is shown on the right y axis). **Panel D** shows the percentages of patients with LOCA who carried an *FGF14* (GAA) $_{250}$ repeat expansion in the French Canadian (40 of 66 index patients), German (42 of 228), Australian (3 of 20), and Indian (3 of 31) cohorts. **Panel E** shows repeat-length variation across 15 maternal and 15 paternal meiotic events involving alleles of (GAA) $_{250}$ repeats.

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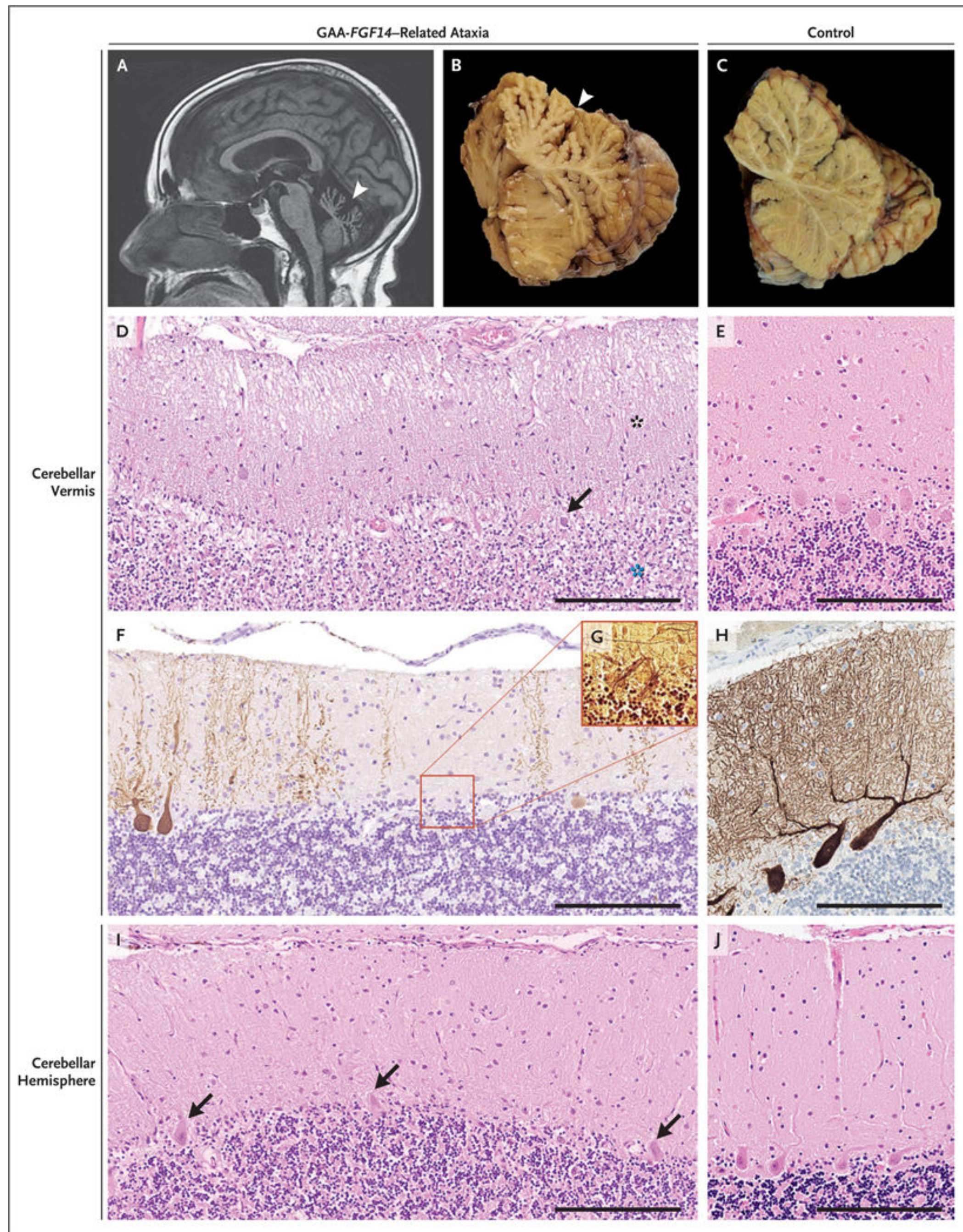

Figure 3. Imaging and Neuropathological Findings in Patients with GAA-*FGF14*–Related Ataxia.

Panel A shows severe vermis atrophy (arrowhead) on sagittal T1-weighted magnetic resonance imaging in a female patient at 88 years of age. **Panel B** shows a midsagittal section of the postmortem cerebellum of the same patient at 94 years of age, in which anterior vermis atrophy is visible (arrowhead); **Panel C** shows an age-matched control for comparison. **Panel D** shows a hematoxylin and eosin–stained section of the cerebellar vermis in the patient, and a control is shown for comparison in **Panel E**. Widespread loss

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of Purkinje cells, with a shrunken appearance of rare residual Purkinje cells (arrow), a gliotic and rarefied molecular layer (black asterisk), and reduced numbers of cells in the granule-cell layer (blue asterisk) can be seen in the patient. **Panels F and H** show calbindin immunohistochemical analysis of the vermis from the same patient with GAA-*FGF14*–related ataxia and a control, respectively. Severe loss of Purkinje cells with markedly rarefied dendritic network in the molecular layer is seen in the patient, whereas in the age-matched control, the Purkinje cells show dense dendritic arborization in the molecular layer. In the inset shown in **Panel G**, Purkinje-cell loss is also evident with Bielschowsky tinctorial silver stain, which highlights the processes of basket cells, resulting in an "empty basket" appearance. **Panels I and J** show the cerebellar hemisphere of a patient with GAA-*FGF14*–related ataxia and a control, respectively; in the patient, the Purkinje cells are reduced in number (arrows), but the molecular layer shows less prominent gliosis and the cell density in the granule-cell layer is much better preserved than in the vermis. All staining was performed with appropriate negative and positive controls. Pathological findings in the cerebellum were similar in the two French Canadian patients with GAA-*FGF14*–related ataxia for whom postmortem tissue was available. The scale bar in Panels D through F and Panels H through J indicates 100 μm.

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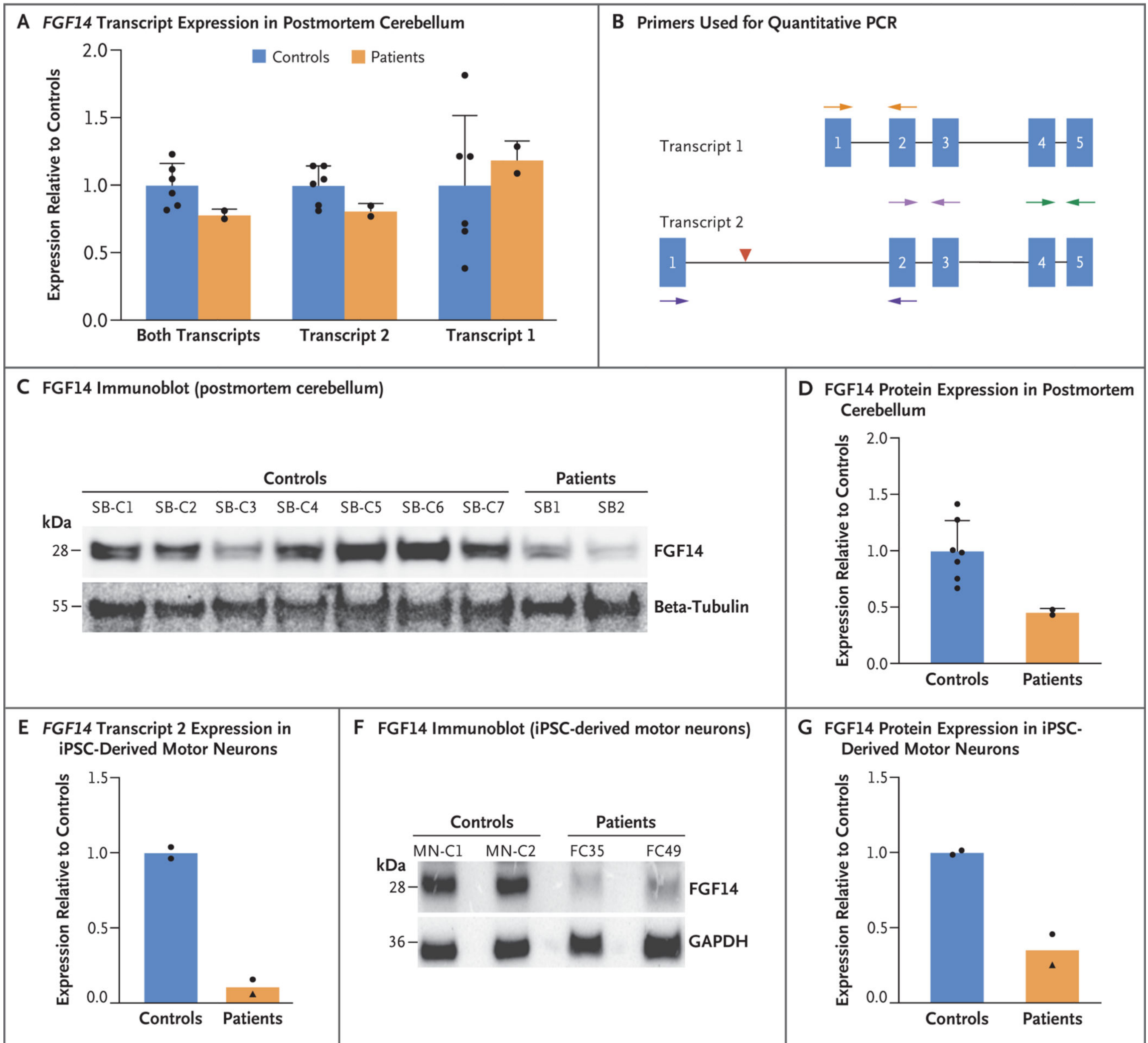

Figure 4. *FGF14* Expression and Protein Levels in Cerebellum and iPSC-Derived Motor Neurons.

Panel A shows the relative expression of *FGF14* transcript variant 1 (NM_004115.4) and transcript variant 2 (NM_175929.3) mRNA, both together and individually, in the postmortem cerebellar cortex of six controls and two patients, normalized by geometric averaging of the expression of five housekeeping genes (*ACTB*, *HPRT1*, *YWHAZ*, *RPL13*, and *UBE2D2*) as assessed by quantitative PCR. Values shown are the ratio of the mean expression relative to the mean among controls. Bars indicate the mean, T bars the standard deviation, and black dots the data distribution. **Panel B** shows a map of the primers used in quantitative PCR experiments with postmortem cerebellum and induced pluripotent stem-cell (iPSC)–derived motor neurons in relation to the exons of both *FGF14* transcripts. Orange arrows indicate the primers used for the transcript 1 assay, dark purple arrows the

primers used for the transcript 2 assay, and green arrows and light purple arrows the primers used for total *FGF14* expression assays in postmortem cerebellum and iPSC-derived motor neurons, respectively. The position of the GAA repeat expansion in intron 1 of transcript 2 is indicated by a red arrowhead. **Panel C** shows a representative FGF14 immunoblot of protein extracts from postmortem cerebellar cortex specimens from seven controls and two patients. Beta-tubulin was used as loading control. Western blot analysis was repeated independently three times, with similar results. **Panel D** shows the mean expression ratios of FGF14 protein in postmortem cerebellar specimens from seven controls and two patients, measured across three independent replicate immunoblots. All ratios were normalized to beta-tubulin and expressed relative to controls. Bars indicate the mean, T bars the standard deviation, and black dots the data distribution. **Panel E** shows the relative expression of *FGF14* transcript 2 in iPSC-derived motor neurons of two controls and two patients, normalized to GAPDH, as assessed by quantitative PCR. Relative quantification was computed by the $2^{-\quad Ct}$ method, with the use of the mean value among controls as calibrator; values are represented as the ratio of the mean expression relative to the mean among controls. Bars indicate the mean, and black dots and triangle the data distribution. **Panel F** shows a representative FGF14 immunoblot of protein extracts from induced motor neurons from two controls and two patients. GAPDH was used as loading control. Western blot analysis was repeated independently twice, with similar results. **Panel G** shows the mean expression ratios of FGF14 protein in induced motor neurons from two controls and two patients. All ratios were normalized to GAPDH and expressed relative to controls. Bars indicate the mean, and black dots and triangle the data distribution. Black triangles in Panels E and G indicate a patient who was homozygous for $(GAA)_{300}$ expansions.

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Table 1.

Characteristics of Patients with GAA-*FGF14*–Related Ataxia.*

Characteristic	French Canadian (N = 68)†	German (N = 48)‡	Australian (N = 3)	Indian (N = 3)	Overall (N = 122)
Male sex — no. (%)	32 (47)	24 (50)	3 (100)	3 (100)	62 (51)
Inheritance — no./total no. (%)					
Sporadic	10/68 (15)	22/44 (50)	2/3 (67)	2/3 (67)	36/118 (31)
Familial	58/68 (85)	22/44 (50)	1/3 (33)	1/3 (33)	82/118 (69)
Episodic features — no./total no. (%)	47/67 (70)	6/48 (12)	1/3 (33)	2/3 (67)	56/121 (46)
Mean age at onset of episodes — yr	54±14	62±7	48	67	55±13
Mean age at onset of permanent ataxia — yr	59±12	60±11	57±11	62±9	59±11
Signs and symptoms — no./total no. (%)					
Nystagmus					
Downbeat nystagmus	43/65 (66)	5/48 (10)	0/3 (0)	2/3 (67)	50/119 (42)
Gaze-evoked horizontal nystagmus	37/65 (57)	25/48 (52)	2/3 (67)	1/3 (33)	65/119 (55)
Diplopia or visual blurring	39/66 (59)	16/48 (33)	0/3 (0)	2/3 (67)	57/120 (48)
Cerebellar dysarthria	39/68 (57)	21/44 (48)	2/3 (67)	1/3 (33)	63/118 (53)
Gait ataxia	65/68 (96)	42/44 (95)	3/3 (100)	3/3 (100)	113/118 (96)
Appendicular ataxia	62/68 (91)	27/44 (61)	3/3 (100)	2/3 (67)	94/118 (80)
Vertigo or dizziness	21/64 (33)	10/44 (23)	0/3 (0)	2/3 (67)	33/114 (29)
Postural tremor	6/60 (10)	11/48 (23)	0/3 (0)	1/3 (33)	18/114 (16)
Cerebellar atrophy on MRI — no./total no. (%)	28/44 (64)	34/41 (83)	2/3 (67)	3/3 (100)	67/91 (74)

* Plus–minus values are means ±SD. Clinical data from 122 of the 128 patients with GAA-*FGF14*–related ataxia are included. Six patients (2 male and 4 female) were excluded because clinical data of sufficient quality were not available. MRI denotes magnetic resonance imaging.

† Data on age at onset of episodes of ataxia were missing for 2 patients, and data on age at onset of permanent ataxia were missing for 2 patients.

‡ Data on age at onset of permanent ataxia were missing for 2 patients.