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GJC2 promoter mutations causing Pelizaeus-Merzbacher-like disease

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Abstract

Objective—Pelizaeus-Merzbacher-like disease is a rare hypomyelinating leukodystrophy caused by autosomal recessive mutations in *GJC2*, encoding a gap junction protein essential for production of a mature myelin sheath. A previously identified *GJC2* mutation (c.-167G>A) in the

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*Dr Inoue and Leo Gotoh share the role of first author in this publication
#Dr Hobson and Dr Vanderver share the role of senior author in this publication

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Authorship and Contributions: Leo Gotoh, Ken Inoue, Grace Hobson and Adeline Vanderver took primary responsibility for writing and revising the paper. Leo Gotoh, Yu-ichi Goto and Ken Inoue performed experiments on promoter region function. Guy Helman, Sara Mora, Kiran Maski, Janet S. Soul, Miriam Bloom, Sarah Evans and Adeline Vanderver examined, counseled and provided patient specific information, Ljubica Caldovic and Grace M. Hobson provided critical review of sequencing results and in silico analyses.

Conflict of Interest: Sara Mora and Dr. Grace Hobson report that they provide some services for A. I. duPont Molecular Diagnostics Laboratory. They could not determine whether a third party paid for diagnostic testing of the patients in the work under consideration. In any case, the patients were recruited into the study after the diagnostic testing was done (conception and planning of the study was done after the diagnostic testing), but the paper mentions the diagnostic test results. Dr. Hobson also reports grants from the NIH. Otherwise, the authors report no conflict of interest.

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promoter region is hypothesized to disrupt a putative SOX10 binding site; however, the lack of additional mutations in this region and contradictory functional data have limited the interpretation of this variant.

Methods—We describe two independent Pelizaeus-Merzbacher-like disease families with a novel promoter region mutation and updated in vitro functional assays.

Results—A novel *GJC2* mutation (c.-170G>A) in the promoter region was identified in Pelizaeus-Merzbacher-like disease patients. In vitro functional assays using human *GJC2* promoter constructs demonstrated that this mutation and the previously described c.-167G>A mutation similarly diminished the transcriptional activity driven by SOX10 and the binding affinity for SOX10.

Interpretation—These findings support the role of *GJC2* promoter mutations in Pelizaeus-Merzbacher-like disease. *GJC2* promoter region mutation screening should be included in the evaluation of patients with unexplained hypomyelinating leukodystrophies.

Keywords

Leukodystrophy; Glia; Myelin; GJC2; Pelizaeus-Merzbacher

1. Introduction

Hypomyelinating leukodystrophies are a rare cause of disease of the central nervous system (CNS) characterized by abnormal myelin formation.[1] The prototype condition for hypomyelinating leukodystrophies is Pelizaeus-Merzbacher disease (PMD) (OMIM 312080)., an X-linked condition[2] that is due to a mutation in the proteolipid protein 1 gene (*PLP1)* (OMIM 300401). Pelizaeus-Merzbacher-like disease (PMLD) (OMIM 608804) is a clinically similar disease without detectable abnormalities within the *PLP1* gene. PMLD is instead an autosomal recessive hypomyelinating leukodystrophy that was shown to be caused by mutations in the gap junction protein gamma-2 gene (*GJC2*) (OMIM 608803) that encodes the connexin 47 protein (Cx47), a connexin family member and gap junction protein important in astrocytes and oligodendrocytes.[3, 4] Mutation of *GJC2* does not allow Cx47 to reach the membrane, resulting in loss of function.[5] Additionally, the *GJC2* promoter region contains SOX10 transcriptional factor binding sites, which allow for SOX10 to play a role in myelin formation.[5]

More than twenty different coding mutations have so far been identified in the *GJC2* coding region.[2, 4, 6-11] An additional mutation, c.-167A>G, was identified in the putative promoter region in individuals with the phenotype of PMLD.[3, 5, 12, 13] This promoter mutation was first identified in the homozygous state, has now been reported in 15 individuals from 5 families,[3, 5, 12, 13] and has additionally been found in two patients[12] in the heterozygous state with another previously published mutation[7] within the coding sequence of *GJC2.* There is evidence suggesting that some c.-167A>G cases arose from a single founder[3, 13] and this mutation is thought to account for nearly a third of *GJC2*-PMLD phenotypes.[13] *GJC2* mutations account overall for only 10% of unsolved cases of hypomyelination, suggesting that mutations in *GJC2* and its promoter region at the SOX10 binding site are a rare cause of this phenotype.[6] Mutation c.-167A>G was demonstrated to

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result in decreased SOX10 dependent transcription of the luciferase reporter gene in constructs containing mouse *Gjc2* promoter region.[5] However, previous studies using the human *GJC2* regulatory region did not show that the c.-167A>G mutation disrupts the SOX10-dependent transcription.[12]

Here we present two PMLD-affected individuals with a novel homozygous mutation (c.-170A>G) in the SOX10 binding site within the *GJC2* regulatory region. We also demonstrate that both the c.-167A>G and c.-170A>G mutations reduce transcription of human *GJC2* using a new luciferase reporter assay. Together, these studies further our understanding of the underlying causes of PMLD and the role of SOX10 in regulation of *GJC2* in CNS myelin formation.

2. Materials and Methods

2.1 Clinical material collection and evaluation

Individual 1 and his unaffected brother were identified prospectively, as part of evaluation of individuals with unsolved leukodystrophies in the IRB-approved Myelin Disorders Bioregistry Project at Children's National Medical Center. Individual 2 and her unaffected family members were enrolled in an IRB-approved research protocol at Nemours Alfred I. duPont Hospital for Children. Affected individuals were examined by author AV (1) and authors JSS and KM (2). Informed consent was obtained. PMLD molecular diagnostic testing was performed in the Molecular Diagnostics Laboratory at the Nemours Alfred I. duPont Hospital for Children.

2.2 Clinical Scoring of PMLD affected by promoter region mutations

Existing literature was reviewed for cases of *GJC2* promoter region mutations, and where clinical data was sufficient, cases were scored according to the motor function score previously used in the PMD and PMLD patient groups[1, 14].

2.3 Bioinformatic analysis of *GJC2* promoter region

The UCSC human genome browser was queried to identify *GJC2* on chromosome 1, intron-exon boundaries, sequence conservation and predicted transcription factor binding sites. Transcription start site (TSS) database[15-17] was queried with the *GJC2* UniGene identifier Hs.100072 to identify *GJC2* TSS. Additional TSS were identified by aligning human *GJC2* ESTs with the human genome (Supplement 1).

2.4 In vitro functional analyses of mutations in the *GJC2* promoter

We generated a luciferase reporter plasmid pGL4-hGJC2_SS-WT, which contains a 384 bp DNA fragment from human *GJC2* promoter. Mutations c.-167A>G and c.-170A>G were introduced at the putative SOX binding site to evaluate their effect on the *GJC2* promoter activity. Human SOX10 expression plasmid pCDNA-hSOX10, and luciferase reporter plasmids were co-transfected into human glioblastoma cells, U138, or human embryonic kidney cells, HEK239 (see Supplementary Materials and Methods). SOX10 binding ability of *GJC2* promoter regions was determined using electro-mobility shift assay (EMSA), as previously described[5] (Supplement 1).

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3. Results

3.1 Clinical Data

Case histories are detailed in supplemental data (Supplement 2) and in Table 1. Both affected individuals presented in the newborn period with congenital nystagmus, and had significant motor delays in early childhood. Neither individual gained independent walking, but both walked supported for some time. Receptive language appeared relatively spared, as with cognition, but expressive language was severely limited by dysarthria. Both individuals had a combination of pyramidal and extrapyramidal motor abnormalities and abnormal oculomotor function on physical examination. In both cases, neuroimaging using MRI (Figure 1 A and B) demonstrates increased T2 and isointense T1 white matter signal consistent with hypomyelination, along with the brainstem signal abnormalities classically seen in PMLD.[19]

Diagnostic testing of affected individuals 1 and 2, including sequence analysis of *GJC2*, identified a homozygous variant (c.-170A>G) in the regulatory region of *GJC2*. Parental testing of the region for both individuals was not available, though an unaffected brother of individual 1 was heterozygous for the variant.

Clinical data from patients with *GJC2* promoter region mutations in the literature suggest that promoter region mutations may result in a milder phenotype than that commonly reported for PMLD patients in general.[14] Patients with c.-167A>G mutations achieved walking with support (three cases) or autonomous walking (13 cases). [3, 5, 12, 13] though in most cases independent ambulation was ultimately lost. In one case data was insufficient to determine best motor function. Our two patients, who achieved walking with support, but never walked independently, are similar to these previously reported cases.

3.2 Bioinformatic analysis of the *GJC2* regulatory region

Bioinformatic tools were used to determine the location of the c.-170A>G variant in the human *GJC2* gene. The human *GJC2* gene spans approximately 19 kb and consists of two exons. Exon 1 is non-coding while exon 2 contains part of the 5′-UTR, the entire connexin 47 coding sequence and the 3′-UTR. The c.-170A>G variant resides within non-coding exon 1 (Figure 2). Human *GJC2* exon 1 may contain two Sox10 transcription factor binding sites[5, 20, 21], as predicted in the mouse *Gjc2* gene, as well as various positions of predicted transcription start sites (TSS) (Figure 2B-D). Both the c.-170A>G variant, identified in this study, and the previously identified c.-167A>G mutation[5] reside in the region of the human gene that corresponds to one of the two mouse Sox10 transcription factor binding sites (Figure 2C-D and Supplemental Figure 1), which are highly conserved in mammals. Positions of the c.-170A>G and c.-167A>G variants within predicted Sox10 binding site and relative to predicted TSS (Figure 2B-D) suggest that these two variants reside either in the promoter or in the 5′-UTR of the human *GJC2* gene. The c.-170A>G variant was not found in either the latest release of dbSNP (version Build 139, Oct 25 2013) or in sequences of 1094 genomes sequenced in the 1000 Genomes Project.

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3.3 Luciferase assays

We utilized a 384 bp minimal *GJC2* promoter construct to determine the effect of mutations in the putative SOX10 binding site using U138 cells, which do not express endogenous SOX10. We observed increased activation of the wild-type *GJC2* promoter activity in U138 cells when SOX10 is co-expressed. When c.-167A>G and c.-170A>G mutations were introduced, this activation was significantly diminished to a level similar to the mutD construct in which one of the two SOX10 binding sites was completely disrupted (Figure 3 A). We also observed similar results in HEK293 cells (Supplemental Figure 2). These findings suggested that both c.-167A>G and c.-170A>G mutations are similarly deleterious. It should be noted that the use of non-glial HEK293 cells resulted in much smaller fold changes than in U138 cells, and the relative activity of c.-167A>G mutant was not significantly different from WT when it was compared to basal activity of WT promoter construct (Supplemental Figure 1). We also tested some longer promoter constructs which extended to the upstream region, including one that Combes et al. reported[12] (Supplemental Figure 3). However, we did not observe efficient up-regulation of transcriptional activities of those promoters in the presence of SOX10, as reported.[12]

3.4 EMSA

The effect of mutations on the SOX10 binding ability was determined by EMSA. A 33 bp probe containing wild type human SOX10 binding site showed strong SOX10 binding (Fig 3B,C), as observed for the corresponding region of the mouse *Gjc2* promoter[5]. Addition of SOX10 antibody revealed a super shifted band, indicating specificity of the binding. Both c.-167A>G and c.-170A>G mutations completely abolished SOX10 binding to the mutated SOX10 binding sites, similar to the absence of SOX10 binding to its mutated consensus binding site (mutD).

Discussion

In this report, we identified a new c.-170 A>G mutation in the *GJC2* promoter region in two independent families with PMLD. The mutation is located in the putative SOX10 binding site, which lies within a highly conserved segment and is predicted to play an important role in *GJC2* transcription. Previously, another promoter mutation, c.-167A>G, was identified in multiple families. Although the c.-167A>G mutation was shown to be deleterious using well-studied mouse promoter constructs, another functional study using human promoter constructs revealed that this alteration had no effect on transcription activity, leading to contradictory interpretation on the effect of the c.-167A>G sequence change. There had been only one promoter mutation, c.-167A>G, reported to cause PMLD, leading to ambiguous interpretation of this alteration. The identification of the c.-170A>G mutation, yet another alteration that putatively disrupt the same SOX10 binding site, in two patients from two families with PMLD, is supportive of the pathogenic role of these sequence alterations in PMLD.

The clinical picture in these two individuals is similar to that previously described in PMLD caused by promoter region mutations. Published PMLD patients with promoter region mutations appear to have a milder course than PMLD overall[14] with the greatest majority

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achieving independent ambulation, and all achieving at least supported walking, though this is typically later lost. There are currently too few patients, however, to know whether there is a correlation between promoter region mutations and a milder disease course and this requires further study.

In order to clarify the role of *GJC2* promoter mutations, we performed functional assays of the two mutations, the c.-170A>G identified in this study and the c.-167A>G, using a minimal promoter construct to study the transcription activity of the human promoter. We demonstrated that both mutations similarly reduced the transcription activity in the human promoter in U138 glioblastoma. The reduced, but not completely abolished transcription activity may allow low level of Cx47 expression, presumably resulting in a relatively mild phenotype in these two patients[5]. In our in vitro transcriptional assays, we utilized a 384 bp minimal promoter fragment, which was markedly activated in the presence of SOX10. The use of the pGL4 reporter system, which eliminated non-specific binding of transcription factors to the vector backbone, may have improved detection of specific transcriptional activities. Longer constructs containing the upstream region, including one that Combes et al. reported[12], did not show efficient up-regulation of transcriptional activities in the presence of SOX10 (supplemental Fig 2). The reason for this difference is unknown, but it is possible that these upstream regions may contain repressor binding elements that diminish the transcriptional activity in the cell lines utilized in our studies. Further studies will be necessary to clarify this point. In conclusion, c.-170A>G and -167A>G mutations disrupt the SOX10 binding to the human *GJC2* promoter, which facilitates normal transcriptional activation of *GJC2*, presumably resulting in reduced expression of Cx47 that is important for the maintenance of myelinating oligodendrocytes

This study has provided two important findings that demonstrate that the *GJC2* promoter mutations can cause PMLD. First, we identified a second alteration in the SOX10 binding site, c.-170A>G, in PMLD patients. Second, functional assays of these two mutations using a minimal promoter construct demonstrated that both mutations similarly reduced the transcription activity in the human promoter (Figure 3). Together with high conservation in mammalian species (Figure 2), these findings suggest that both PMLD-causing mutations in the SOX10 binding site are likely deleterious and probably result in a down-regulation of the *GJC2* promoter activity. These two findings together provide genetic confirmation of pathogenicity of mutations in the SOX10 binding site in the *GJC2* promoter region as causative of PMLD. *GJC2* promoter region mutation screening should therefore be included in the evaluation of patients with unexplained hypomyelinating leukodystrophies

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- A homozygous mutation in the GJC2 regulatory region was found in two cases of PMLD
- c.170A>G is the second promoter mutation found in the SOX10 binding site of *GJC2*
- Reduced transcription is shown by both mutations through a luciferase reporter assay
- PMLD-causing mutations may result in profound down-regulation of the *GJC2* promoter
- We provide genetic confirmation of pathogenicity by promoter mutations in PMLD

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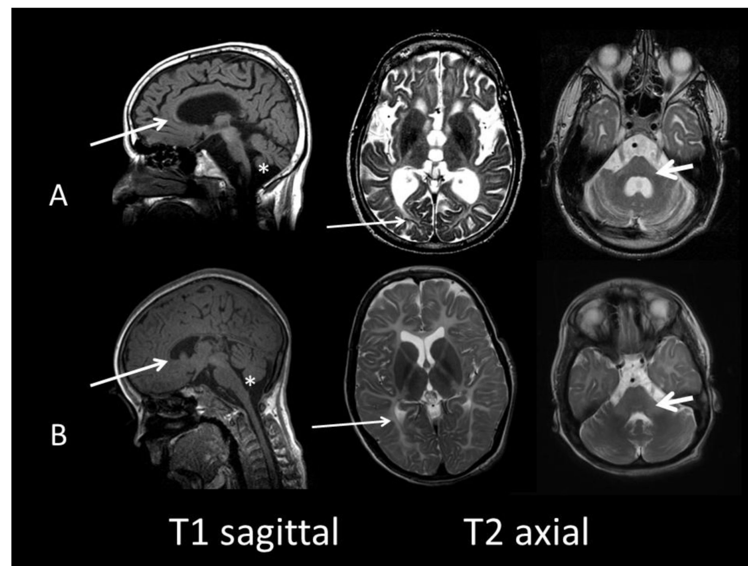

Figure 1. Imaging results

A. Neuroimaging of individual 1 at age 32 revealed severe loss of the cerebral periventricular white matter with associated prominence of the supratentorial sulci (thin arrow T2 Axial) with a diffusely thin corpus callosum (thin arrow T1 sagittal). There was, to a lesser degree, volume loss of the cerebellum with associated mild prominence of the sulci (asterisk). There was T2 hyperintensity of the residual cerebral white matter diffusely suggesting a persistent deficit of myelin. **B.** A brain MRI performed on individual 2 at 2 years of age in Brazil reportedly showed abnormal signal in the white matter, thalamus and basal ganglia. Brain MRI scans at age 8 years showed T2 prolongation in the white matter throughout the entire brain (thin arrow T2 Axial) and brainstem (T2 axial thick arrow), with a diffusely thin corpus callosum (thin arrow T1 sagittal). Spectroscopy demonstrated an increased N-acetyl aspartate (NAA) to choline/ creatine peak (not shown) in affected white matter. The cerebral and cerebellar sulci were mildly prominent, with no significant change over the two-year interval (asterisk).

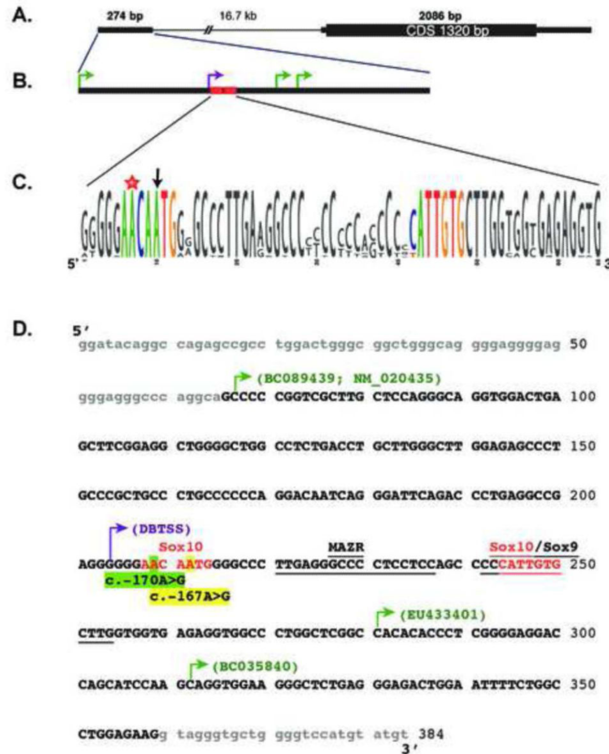

Figure 2. Map of the human GJC2 gene, positions of the c.-170A>G and c.167A>G variants, and transcription factor binding sites

A. Two exons (solid boxes) and an intron (line) of the human GJC2 gene. Sizes of exons, an intron and coding sequence (CDS) are shown. **B.** Map of the non-coding exon 1. Red boxes indicate positions of two predicted Sox10 binding sites in the mouse *Gjc2* gene. Angled arrows indicate predicted Transcription start sites (TSS); Purple arrows are TSS predicted in the database of TSS (DBTSS); Green arrows are TSS predicted based on the alignment of human GJC2 ESTs. Locations of TSS were determined based on sequencing of whole length mRNA in the TSS database and on the alignment of human *GJC2* ESTs with genomic sequence in mice. **C.** LOGO alignment of sequences from 16 mammals (see S1) that include two Sox10 binding sites and flanking sequences. Sox10 binding sites are shown in colored letters. Variant c.-170A>G is indicated with the star. Variant c.-167A>G is indicated with down-arrow. The size of letters in the alignment corresponds to the degree of conservation at that position in the alignment. All sequences from 16 mammals have an A at positions that correspond to the c.-170A>G and c.-167A>G variants in the human *GJC2* gene. **D.** Sequence of exon 1 of the human GJC2 gene (upper case) and its flanking sequences (lower case and gray typeface). Predicted TSS are shown as angled arrows. Accession numbers of corresponding human GJC2 ESTs are in parenthesis. Two predicted Sox10 binding sites are shown in red. Predicted Sox9 and MAZR transcription factor binding sites are underlined (from the transcription factor binding site track of the UCSC genome browser). Variants c.-170A>G and c.-167A>G are highlighted in green and yellow, respectively. In addition to the two predicted Sox10 binding sites, *GJC2* also has predicted Sox9 and MAZR transcription factor binding sites (Figure 2D).

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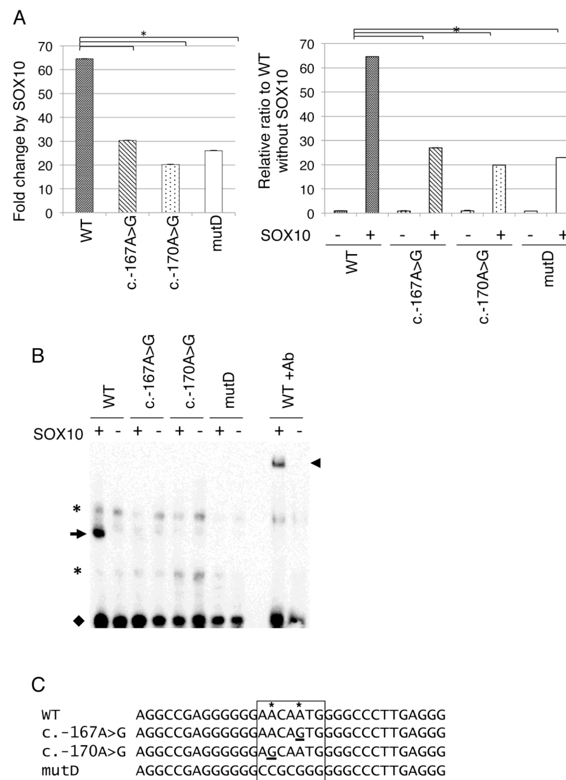

Figure 3. *In vitro* functional assays of GJC2 mutations

A. Transcription activities determined by luciferase reporter assays in U138 cells. Left panel shows fold changes of transcriptional activity of each promoter construct between co-transfection of pCDNA-hSOX10 and an empty plasmid, pCDNA3.1. Right panel shows luciferase activities as the relative ratio above the mean activity from transfections with WT promoter and an empty plasmid, which was arbitrarily set as 1. A renilla luciferase reporter plasmid pRL (Promega) was used as an internal control to normalize transfection efficiency. The asterisk indicates the statistical significance of $p<0.001$ by ANOVA. The experiment was repeated three times with 3 independent samples per experiment and representative results from one experiment are shown. **B**. EMSA showing SOX10 binding to each probe tested, as listed in C, in the presence (+) and absence (−) of SOX10. Arrow indicates the specific binding of SOX10 to the wild-type (WT) probe. Arrowhead indicates supershift by addition of anti-SOX10 antibody. Rhombus indicates free probes. Asterisks indicate nonspecific bands. The experiment was repeated three times and a representative result is shown. **C.** Oligo probes used in B. A square indicates putative SOX10 binding site. Asterisks show the position of mutations and nucleotide alterations are underlined.

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Table 1

Clinical Features of Affected Individuals

Finding	Individual 1	Individual 2
Gender	Male	Female
Ethnicity	Polish descent	Portuguese descent
Age at presentation	Newborn	Newborn
Current age	39 years	9 years
Presenting sign	Congenital nystagmus	Congenital nystagmus
Initial motor development	Crawling at 19 months and cruising by 24 months. He never established independent walking	At age 6 years she was unable to sit or stand without assistance even though she could commando crawl
Onset of motor deterioration	Gradual deterioration in early childhood related to spasticity	No episodes of deterioration
Loss of supported walking ability	Loss of supported walking around 4-5 years	At age 9 years still could ambulate with a walker with truncal support
Spasticity	+	+
Rigidity	+	+
Dystonia	+	+
Ataxia	–	+
Tremor	–	+
Choreoathetosis	–	–
Dysarthria	+	+
Scoliosis	+	+
Oculomotor abnormalities	Rotatory nystagmoid eye movements	Impaired smooth pursuit, continuous, pendular nystagmus and intermittent exotropia
Vision	Severe myopia since early childhood	Severe myopia and astigmatism since early childhood
Hearing	Normal	Normal
Cognitive decline	+	NA
Language development	At 9 years of age, he was reported to use 3 to 4 word sentences, but that ability was diminished over time by dysarthria. Currently only able to say yes or no.	At 9 years she had 6 sign language words and was able to say 2 words with dysarthric speech.
Epilepsy	+ mild and easily controlled	none