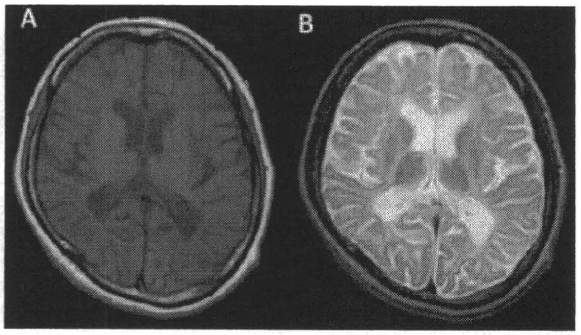


FIGURE 1: Magnetic resonance imaging of the cerebrum. (A) T1-weighted image of the proband at 20 years shows cerebral atrophy with ventricular dilatation and widening of a subarachnoidal space. Disappearance of contrast between cortex and white matter, which suggested incomplete myelination throughout the cerebrum, was evident. (B) T2-weighted image reveals diffuse hyperintensity in the white matter, suggesting the arrest of myelination. Note that the inner capsule, which is usually myelinated in the neonate, was not myelinated in this patient.

mal. Molecular examinations excluded *PLP1* exonic mutations, large duplications, and deletions.

Informed consent was obtained from the patient and family members in accordance with human study protocols approved by the institutional review board of Kanagawa Children's Medical Center. Genomic DNA was extracted from peripheral lymphocytes. A genome-wide single nucleotide polymorphism (SNP) genotyping was undertaken for III-1, III-3, IV-1, IV-2, V-1, and V-2 (Fig 2A) using the GeneChip Human Mapping 10K Array *X*ba 142 2.0 (Affymetrix, Santa Clara, CA) containing 10,204 SNPs according to the manufacture's protocols (Supplementary Materials and Methods). Polymerase chain reaction and DNA sequencing are described in the Supplementary Materials and Methods (Supplementary Table 1).

Mammalian cell expression plasmids for the wild-type and E189X mutant human *SOX10* cDNA were reported previously.[12] Luciferase reporter plasmids containing mouse *Gjc2* promoters (kindly provided from Dr M. Wegner) were utilized

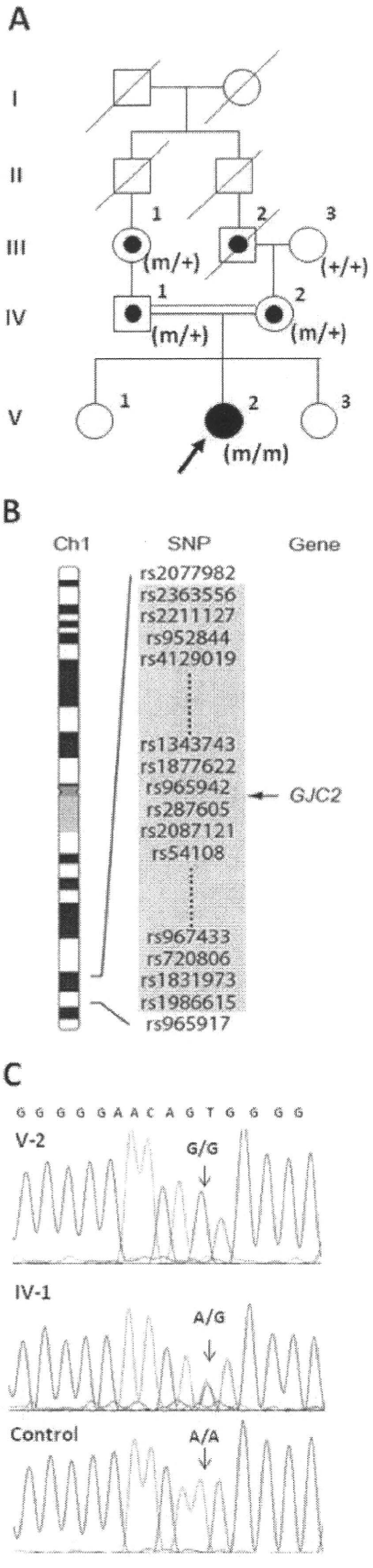
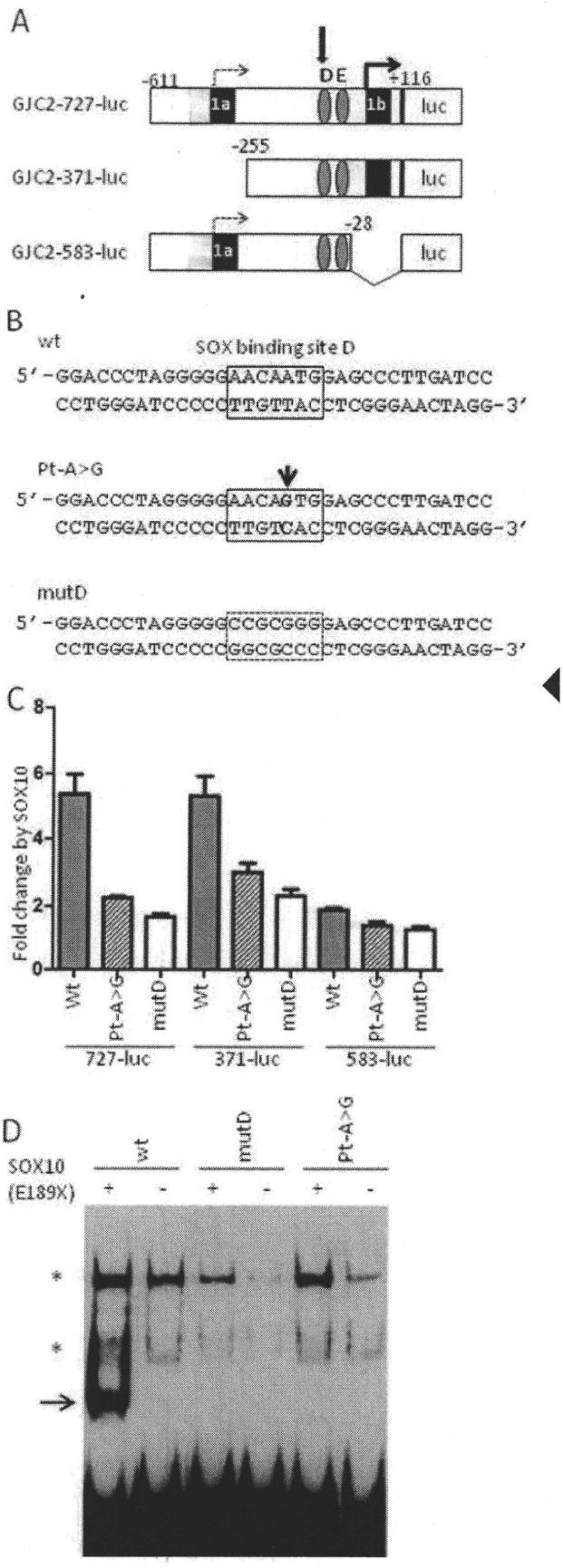


FIGURE 2: Family pedigree, largest region of interest on chromosome 1, and the *CJC2* mutation. (A) Pedigree of the Pelizaeus-Merzbacher-like disease family with the proband *(filled circle with arrow)*. DNA from III-1, III-3, IV-1, IV-2, V-1, and V-2 were used for single nucleotide polymorphism (SNP) genotyping. Carriers are indicated as circles with black dots. m = mutant allele; + = wild-type allele. (B) The largest region of interest by homozygosity SNP mapping at 1q41-q42.2. The homozygous interval is shown as a shaded square with SNP identifiers. The location of *GJC2* is shown with an arrow. The region between rs2077982 and rs965917 was 18.2 Mb in size (University of California, Santa Cruz genome browser coordinate, chromosome 1: 215150317-233384165, February 2009 version). (C) Sequencing chromatograms from the patient (V-2, top), a carrier (IV-1, middle), and a normal control (bottom). The c.-167A>G mutation in the promoter region of *CJC2* is shown with arrows.



for site-directed mutagenesis (see Supplementary Materials and Methods). We measured *GJC2* transcriptional activity by luciferase reporter assays using human glioblastoma U138 cells (see Supplementary Materials and Methods). SOX10 binding affinity was determined by electrophoretic mobility shift assay (EMSA) using synthetic oligonucleotide probes and nuclear extracts from HeLa cells transfected with pCMV-SOX10-E189X, as previously described.[13]

Results

The largest region with homozygosity identified by SNP genotyping on chromosome 1q42.13 was our primary focus for candidate gene scanning (see Fig 2B, Supplementary Table 2, Supplementary Fig 1). Among 115 refseq genes mapped within this region, 34 gene products were identified from mouse whole brain proteomics studies (Supplementary Table 3).[14,15] After we sequenced all coding regions and intron-exon boundaries of these 34 genes to exclude any disease-causing mutations, we extended our analysis to promoter regions. We found a homozygous mutation, c.-167A>G, in the proximal promoter re-

FIGURE 3: Functional consequence of the c.-167A>G point mutation in the *GJC2* promoter. (A) Schematic diagram of the luciferase reporter constructs of mouse *Gjc2* promoter region utilized in this study. Exon 1b contains the major transcription start site *(thick arrow)*, whereas exon 1a contains the minor site *(dotted arrow)*. GJC2-727-luc contains a full proximal promoter, whereas GJC2-371-luc lacks exon 1a and the upstream portion and GJC2-583-luc lacks exon 1b. Two SOX10 binding site, D and E, are shown as shaded ovals with a thick arrow pointing to site D, where the mutation was identified. (B) Sequences of the probes used for electrophoretic mobility shift assays (EMSAs). Top: *wt* probe containing the wild-type site D *(square)*. Middle: Pt-A>G probe carrying c.-167A>G mutation *(arrow)*. Bottom: mutD probe in which site D was changed to abolish SOX10 binding. (C) Transcriptional activities of different *GJC2* promoter constructs carrying either wt, Pt-A>G, or mutD at site D shown as fold changes obtained by presence or absence of SOX10 determined by luciferase reporter assay. Note that the *wt* constructs for 727-luc and 371-luc, harboring the major start site in exon 1b, were activated by SOX10 >5-fold. In contrast, a much smaller effect was observed when either Pt-A>G or mutD was introduced. The 583-luc constructs, which only harbor a minor transcription start site, remain inactivated by SOX10 regardless of changes in site D. Each bar represents average ± standard deviation. Each experiment was performed 3×, each in triplicate. Results from a representative experiment were shown. (D) DNA binding affinity of each probe (shown in B) was determined by EMSA using nuclear extracts from HeLa cells transfected with plasmid expressing truncated SOX10 protein (E189X) or empty plasmid (as a negative control). The *wt* probe showed a strong binding to E189X SOX10 protein, which retains enhanced DNA binding ability *(arrow)*. In contrast, we observed no binding of the mutant probes, either mutD or Pt-A>G. Asterisks show nonspecific binding. Free probes were observed at the bottom of the picture.

gion of *GJC2* that segregated with PMLD in the family members (see Fig 2A and C) and was absent in 122 normal Japanese chromosomes. Analysis of this region in 10 additional female PMLD patients without mutations in the open reading frame of *GJC2* detected no abnormalities.

Interestingly, this mutation is located within a critical SOX10 binding site (designated as site D) in the syntenic mouse *Gjc2* proximal promoter and diminishes the consensus of the SOX binding sequence (AACAATG to AACAGTG, Fig 3A and B). Based on this, we predicted that this mutation disrupts *GJC2* promoter activity and measured transcription in vitro using a luciferase reporter system. Because the region harboring the mutation is highly conserved across mammals,[16] we introduced this mutation into well-studied mouse *Gjc2* promoter constructs (see Fig 3A and B). The c.-167A>G point mutation in the SOX10 binding site dramatically decreased transcription to levels similar to a completely disrupted SOX10 binding site D (see Fig 3C). These findings suggest that the c.-167A>G point mutation found in our patients results in a diminished *GJC2* transcription.

Based on these results, we hypothesized that this mutation altered SOX10 binding affinity to site D and tested this by EMSA. Because full-length SOX10 has a low binding affinity that is difficult to distinguish from background noise, we used a C-terminus truncation version of SOX10, E189X, which retains the HMG binding domain and has enhanced binding affinity.[16] Introduction of the c.-167A>G mutation into site D resulted in a complete loss of E189X SOX10 binding (see Fig 3D). Therefore, combined with the preceding observations, we find that the c.-167A>G mutation abolishes SOX10 binding to the *GJC2* promoter, resulting in a dramatic attenuation of the *GJC2* transcription.

Discussion

GJC2 encodes Cx47, a member of the connexin family. Connexins are components of gap junctions, intercellular channels that allow ions and small molecules to pass across neighboring plasma membranes. Gap junctions have diverse functions, including the propagation of electrical signals and metabolic cooperation. Two hemichannels, each built up of 6 connexin protein subunits on opposing cell membranes, form the channel. Astrocytes and oligodendrocytes are coupled by gap junctions constructed predominantly of GJC2 (Cx47) and Cx43.[17] Because Cx47 proteins carrying PMLD-causing mutations either fail to reach the membrane or have reduced transport activity, loss of function is likely the mechanism underlying the CNS hypomyelination in PMLD.[7,9] Herein we report the first *GJC2* promoter mutation,[18] c.-167A>G, in a patient with PMLD, and this is associated with allelic transcription failure.

Our female patient had nystagmus, spasticity, and choreoathetosis, clinical symptoms common to PMD and PMDL. However, she attained normal motor and intellectual developmental milestones. Because only $\frac{1}{3}$ (11 of 33) of PMLD patients with *GJC2* mutations have walked unsupported,[2–7] her clinical manifestation was mild and overlaps with that of spastic paraplegia phenotype. Of note, she lost her motor and cognitive abilities within a few years, accompanied by progressive brain atrophy (see Fig 1). Such acute regression has rarely been observed in PMD and is more characteristic of PMLD secondary to *GJC2* mutations.[6]

A recent study showed that SOX10 directly regulates *GJC2* by binding to its proximal promoter.[16] Site D, the SOX10 binding site in which our mutation was identified, plays a predominant role in *GJC2* promoter activity,[16] and the c.-167A>G mutation we identified reduces its affinity for SOX10 and abolishes *GJC2* transcription. These findings suggest that *SOX10* regulation of *GJC2* via site D is essential for proper *GJC2* expression and that its failure causes PMLD. Presumably, the relatively milder clinical phenotype observed in our patient results from reduced but not completely abolished transcriptional activity, allowing translation of a small amount of normal Cx47 protein.

This constitutes the second disorder associated with dysregulation of a SOX10 target gene. Previously, mutations within the *SOX10* binding site of the *GJB1* promoter have been shown to cause demyelinating peripheral neuropathy.

Together the peripheral neuropathy and PMLD provide a partial understanding of the clinical manifestations of PCWH patients. Because these patients have SOX10 mutations,[10] we predict that the expression of both *GJC2* and *GJB1* is impaired. Impaired expression of both of these genes would, at least in part, respectively account for the de-/hypomyelination of the CNS and PNS observed in PCWH. Based on this, we predict that impaired expression of other target genes of SOX10 is responsible for the Hirschprung disease and other Waardenburg features.

In conclusion, we identified the first case of PMLD caused by a mutation in the *GJC2* promoter. Because this mutation disrupts SOX10 regulation of *GJC2* transcription, we hypothesize that SOX10 regulation of transcription plays a major role in nervous system myelination.

Acknowledgments

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Potential Conflicts of Interest

Nothing to report.

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