



Published in final edited form as:

Ann Neurol. 2018 November ; 84(5): 638–647. doi:10.1002/ana.25327.

Loss of *protocadherin-12* leads to Diencephalic-Mesencephalic Junction Dysplasia syndrome

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Abstract

Objective: To identify causes of the autosomal recessive malformation diencephalic-mesencephalic junction dysplasia (DMJD) syndrome.

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Author Contributions

M.S.Z., M.G. and J.G.G. contributed to the conception and design of the study, A.G.-G., M.S.Z., A.O.-C., S.N.S., H.P., H.G., A.K.B., S.K., G.T.A., E.Z.E.-O., K.Y., K.B., H.G., N.P., N.S., D.W.-V., Y.P., R.O.R., B.B.-Z., A.G., N.A., A.O.-C., J.L.S. S.M.L. R.F., J.S., D.M., J.M.-V., D.B., B.C., S.G., and M.G. contributed to the acquisition and analysis of data, A.G.-G., V.S. and J.G.G. contributed to drafting the text and preparing figures, which was edited by all co-authors.

Potential Conflicts of Interest

Nothing to report

Methods: Eight families with DMJD were studied by whole exome or targeted sequencing, with detailed clinical and radiological characterization. Patient-derived induced pluripotent stem cells were derived into neural precursor and endothelial cells to study gene expression.

Results: All patients showed bi-allelic mutations in the non-clustered *protocadherin-12* (*PCDH12*) gene. The characteristic clinical presentation included progressive microcephaly, craniofacial dysmorphism, psychomotor disability, epilepsy, and axial hypotonia with variable appendicular spasticity. Brain imaging showed brainstem malformations and with frequent thinned corpus callosum with punctate brain calcifications, reflecting expression of *PCDH12* in neural and endothelial cells. These cells showed lack of *PCDH12* expression and impaired neurite outgrowth.

Interpretation: DMJD patients have bi-allelic mutations in *PCDH12* and lack of protein expression. These patients present with characteristic microcephaly and frequent abnormal white matter tracts. Such pathogenic variants predict a poor outcome as a result of brainstem malformation and evidence of white matter tract defects, and should be added to the phenotypic spectrum associated with *PCDH12*-related conditions.

Keywords

non-clustered protocadherin; *PCDH12*; neural circuit; epilepsy; intellectual disability; microcephaly; diencephalic mesencephalic junction dysplasia

We reported a new clinical condition, Diencephalic-Mesencephalic Junction Dysplasia (DMJD), characterized by a distinctive brainstem ‘butterfly sign’ on axial CT/MR images. Although the genetic basis was not previously known, clinical data suggested a unique developmental brainstem defect.¹ The ‘butterfly sign’ was attributed to an anterior midbrain cleft contiguous with the third ventricle, along with downward displacement of the diencephalic-mesencephalic junction. In DMJD patients studied with tractography, the corticospinal tract, which originates in the cortical motor area and normally courses medially to project to the contralateral spinal cord, instead coursed laterally and appeared to terminate prematurely. A defect in corticospinal tract function was clinically evidenced by spasticity of the limbs accompanied by quadriparesis. Additionally, thinned corpus callosum in the majority of subjects supported a white matter tract developmental defect. Since the original publication, several reports of additional patients with DMJD have appeared,^{2–4} suggesting a unique radiographically-defined condition, but the genetic basis remained unknown.

Here we analyze two of the originally described DMJD families and found bi-allelic loss-of-function variants in *protocadherin-12* (*PCDH12*). Moreover, we identify six additional families for a total of eight independent families with 14 patients carrying pathogenic variants in *PCDH12*. *PCDH12* belongs to the non-clustered group of the protocadherin family, the largest group within the cadherin superfamily,^{5,6} encoding an 1184 aa glycoprotein with six cadherin domains, a transmembrane domain, and an intracellular domain. *PCDH12* was originally identified in endothelioma cell lines,⁷ where it concentrates at cell-cell junctions and promotes cell adhesion.^{8,9}

Protocadherins are diversified cell-surface proteins with over 70 members that are part of the cadherin superfamily. The protocadherin family is subdivided into the clustered and non-clustered groups based on their genomic organization.^{5,6} Correlated expression patterns of

non-clustered protocadherins suggest roles in neural circuit formation,^{10,11} and several non-clustered protocadherin genes have been implicated in neurological diseases. Pathogenic variants in the X-linked *PCDH19* cause female-specific epilepsy with mental retardation (MIM#300088).^{12,13} *PCDH8*, *-9* and *-10* have been linked to autism,^{14–16} *PCDH7* has been linked to epilepsy,¹⁷ and *PCDH17* variants confer a risk for mood disorders.¹⁸ *PCDH12* variants have been associated with schizophrenia¹⁹ and recently bi-allelic pathogenic variants have been linked to microcephaly, seizures, spasticity with brain calcifications (MISSBC).^{20,21} Our report extends the phenotypic spectrum associated with *PCHD12* mutations and adds to the growing body of neurological disorders linked with altered protocadherins.

Methods

Patient Recruitment

Patients were identified from genetics and/or neurology clinics targeting patients with structural brain diseases or neurodevelopmental disorders in regions of the world displaying elevated rates of parental consanguinity. All patients/families enrolled in IRB approved protocols and provided consent for study. The total cohort includes >6,000 individual families recruited between the period of 2004–2017 presenting with features of intellectual disability, autism-related conditions, microcephaly, structural brain disorders, epilepsy, or neurodegeneration. The cohort was enriched for families with recessive pediatric brain disorders, due to consanguinity (>80% of pedigrees) and multiple affected members (>60% of families). Routine medical records, history, physical and neurological examination as well as evaluation of brain MRI or computed tomography (CT) were carried out as part of the standard clinic evaluation, and several patients underwent both modes of imaging. Determination of callosal abnormalities was based on visual observation by taking into consideration the age of each patient at the time of imaging. Pedigree analysis and blood sampling were pursued in all families, and subjects were selected for exome sequencing based upon a clinically defined neurodevelopmental genetic condition. Sequencing was performed on one or two affected patients or the father-mother-affected trio per family.

Exome Sequencing

Genomic DNA was extracted and subjected to exon capture, sequencing, variant calling, and computational filtering as previously described.²² Briefly, genomic DNA was extracted (Qiagen or Oragene) and subject to exon capture with the Agilent Sure Select Human All Exome 50Mb Kit. Paired-end sequencing was with Illumina HiSeq2000 or HiSeq4000 instruments,²³ resulting in 94% recovery at >10× coverage and 85% recovery at >20× coverage. Genome Analysis Toolkit (GATK) was used for variant identification, and then filtered for homozygous variants using the Varvis software (Limbus Tech, GmbH), to deprioritize alleles with >0.1% frequency in control populations (in-house exome data set of 9,500 individuals, dbSNP and ExAC Exome variant server), occurring outside of homozygous intervals in consanguineous families, or outside of linkage intervals. The remaining variants were ranked by the type of pathogenic variant (nonsense/splice/indel > missense), amino acid conservation across species, and damage prediction programs (PolyPhen and SIFT score).

Cell Transfection

Human full length *PCDH12* was amplified from mammalian gene collection (MGC) fully sequenced cDNA clone, cloned into either FLAG-tagged mammalian expression vector, and mutagenized to incorporate patient mutations. 293T cells (ATTC) were transfected with the FLAG-tagged expression vectors using Lipofectamine 2000 (Thermo Fisher Scientific). Cell extracts were analyzed by Western blotting with primary antibodies against FLAG (Sigma, F7425) or α -tubulin (Sigma, T6074), detected by horseradish peroxidase conjugated secondary antibodies and chemiluminescence (Thermo Fisher Scientific).

Fibroblast Culture, iPSCs, and NPCs Generation

Primary fibroblast cell lines were established from unaffected and affected skin explants of dermal biopsies from Family 1 (1592), according to standard methods. iPSCs were generated as previously described,²⁵ by episomal gene transfer of *cMYC*, *OCT4*, *KLF4*, and *SOX2*. Colonies with healthy appearance (rounded smooth edges) were selected for further cultivation and evaluation. Neural progenitor cells (NPCs) were obtained as previously described,²⁶ with iPSCs cultured in serum free floating embryoid body-like aggregates with 1mM Dorsomorphin (Tocris), 2mM A8301 (Tocris) and kept shaking at 95 rpm for 7 days. Resultant embryoid bodies (EBs) were plated onto Matrigel (BD Biosciences) coated dishes in NBF medium (DMEM/F12, 0.5X N2, 0.5X B27, 20ng/ml bFGF) to yield rosettes, which were dissociated with Accutase (Millipore) after 5–7 days, and resultant NPCs plated onto poly-ornithine/laminin (POL, Sigma) coated dishes in NBF medium. Endothelial cells (ECs) were differentiated as previously described,²⁷ iPSCs were cultured in serum-containing media in a non-adherent petri dish for 13 days. Resultant embryoid bodies (EBs) were dissociated to single cells and incubated with anti-CD31-FITC (BD Pharmingen). FACS analysis to sort CD31-positive cells was performed on a FACS Aria instrument (BD Biosciences) and resultant CD31+ cells were plated onto 1% gelatin coated dishes in EGM-2 medium (Lonza). Experiments were performed with NPCs at passage 4–6 and ECs at passage 3–5. Bright field images were taken in EVOS microscope and processed with Photoshop CS5 (Adobe Systems).

Neurite Length Quantification.

To determine neurite length, NPCs were plated on POL coated dishes and transfected with pEGFP plasmid using Lipofectamine 2000 (Thermo Fisher Scientific). pEGFP tagged NPCs were allowed to extend processes, then analyzed after 24h. Cells were fixed 10min with 4% PFA, washed with PBS, permeabilized with 0.15% Triton X-100, blocked 1h in the same solution with 5% normal donkey serum. Slides were incubated with primary antibody (Nestin; 1:2,000, Millipore) overnight at 4°C and then incubated with fluorescently labeled secondary antibody (Jackson ImmunoResearch) for 2h at room temperature. Fluorescent signal was detected using an Olympus IX51 inverted microscope or Leica SP5 confocal microscope, then images processed with Photoshop CS5 (Adobe). Length of the primary neurite per NPC was measured using FIJI/ImageJ (NIH).

Results

Identification of Pathogenic Variants in *PCDH12*

In order to identify causes of DMJD, we first studied the originally reported families.¹ We performed whole-exome sequence (WES) analysis from two affected individuals in Family 1 (originally described as Family 1592), with prioritization of variants based upon established criteria²⁸ and < 0.1% allele frequencies derived from an in-house exome database from ethnically-matched individuals. We identified six variants of moderate-to-high impact (See Supplementary Table 1). Further filtering of variants for only 'high-impact' identified a single rare homozygous hg19:chr5:141334906delC variant in *PCDH12* (c.2511delG), a 1-bp deletion, leading to a frameshift (p.S838fs*) (Fig 1A,B), which segregated according to a recessive mode of inheritance. Sanger sequencing of the coding region of *PCDH12* in the affected individuals of previously described Family 2 (originally described as Family 1846)¹ identified a hg19:chr5:141334652delAG (c.2765delCT) 2-bp deletion leading to a frameshift (p.P922fs*). Of the three families originally described with DMJD¹ all displayed evidence of the 'butterfly sign', Families 1 and 2 showed lateral ventricles with typical size and Family 1825 showed massively dilated lateral ventricles. Consistent with that phenotypic difference, in Family 1825 no *PCDH12* pathogenic variant was identified. The presumed role of *PCDH12* in cell-cell contact-dependent recognition made it an attractive candidate for DMJD, prompting further investigation.

We identified six additional families with pathogenic variants in *PCDH12*. WES analysis from our cohort of identified six additional *PCDH12* homozygous truncating pathogenic variants. Family 3 and Family 4 both displayed the homozygous 2-bp deletion (c.2765delCT) present in Family 2, and Family 5 presented with a nonsense pathogenic variant hg19:chr5:141336579C>A (c.838G>T) leading to a premature stop codon (p.E280*) (Fig 1A,B). No other prioritized variants segregated in any family (See Fig 1 and Supplementary Tables 2 and 3), leaving *PCDH12* as the top candidate for each family.

A second cohort of 2000 subjects with neurodevelopmental disorders from the Yale Mendelian Sequencing Consortium was analyzed with WES and identified two additional consanguineous families with homozygous deleterious variants in *PCDH12*. Family 6 displayed a hg19:chr5:1413336965G>A (c.452C>T) variant leading to a p.R151* premature stop codon, and Family 7 displayed the same 2-bp deletion (c.2765delCT) found in Families 2, 3 and 4. We identified an additional Moroccan Jewish non-consanguineous Family 8 presenting with a homozygous 1-bp deletion hg19:chr5:141336357delC (c.1060delG), p.Val354fs* (Fig 1A,B). Direct questioning showed no evidence of known shared ancestry for families sharing a common pathogenic variant. No correlation was detected between individual pathogenic variant and overall disease severity. No biases were detected in severity or age of onset based upon patient sex, and all pathogenic variants were fully penetrant without observed phenocopies within the family. Direct Sanger sequence confirmed segregation in all available family members according to strict recessive inheritance with full penetrance. To study the effect of patient pathogenic variants we used point mutagenesis to introduce c.2765delCT (most common variant), c.2511delG (example frameshift), and c.452C>T (example nonsense), in a plasmid containing full length

PCDH12. We found no protein expression after misexpressing mutated *PCDH12* constructs in 293T cells. (Fig 1C). We conclude that pathogenic variants tested result in lack of *PCDH12* expression due to nonsense mediated decay.

***PCDH12* Patients show Microcephaly and Spasticity**

Clinical features of all 14 individuals from the original two families with *PCDH12* pathogenic variants and six new families are summarized in Table 1 and reported in detail in Supplementary Table 4. Recurrent fever of unknown etiology was seen in 64% of patients, with early onset and up to 2 months of age. Epilepsy was seen in 76% of patients with onset usually before 3 months of age and consisted of tonic, atonic, generalized tonic-clonic and focal seizures, partially controlled with anticonvulsant medications. History of recurrent vasomotor instability was prominent in more than half of the patients (57%). The study included ten males and four females, and ages ranged from 18 months to 6 years.

All patients presented with microcephaly and profound psychomotor delay. Head circumference was 1.5 to 8.7 SD below the mean, with almost 80% of patients (78%) in the range of 3 to 6 SD below the mean. Head circumference at birth was available in 7 of 14 patients and ranged from 0.6 to 1.5 SD below the mean, indicating predominantly post-natal progressive microcephaly. Bitemporal narrowing due to severe microcephaly was noted in half of patients. None was able to stand or walk independently and none developed meaningful fine motor skills or language. Most patients displayed severe cognitive impairment and 28% of patients presented with autistic features. Ataxia was present in 42% of the patients. Pyramidal tract signs were evident in all; 64% of patients presented with hyperreflexia while the rest displayed truncal hypotonia (28%), and mild to severe appendicular spasticity were present in 28% of patients. Cranial nerve findings included strabismus (28% of patients), dysphagia (28% of patients), minimal to absent tracking of objects (21% of patients), and squinting (14% of patients). A combination of two or even three of these cranial nerve findings was observed in those more severely affected patients (1-V-2, 2-IV-2, and 3-IV-1). Facial dysmorphism was noted in most of the patients (85%), which included low-set ears, broad prominent nasal bridge, long flat philtrum, broad chin and thin upper lip (Figure 1D). Metabolic profile and echocardiography was normal in all patients evaluated.

Brain imaging was available in 12 of the 14 subjects. Five of the patients underwent only MRI, whereas the other seven had MRI plus CT head imaging. Brain imaging demonstrated a variety of brainstem malformations present in affected individuals in all families; including the DMJD pathognomonic 'butterfly'- like contour of the midbrain on axial sections (Fig 2). Thin corpus callosum was frequently observed in patients, and did not show more severe thinning in older patients, arguing against progressive atrophy (Fig 3). Overall, cerebellum and pons were preserved and ventriculomegaly was present in 57% of patients. Our investigation did not reveal any overt vascular or coagulation phenotypes in patients (Supplementary Table 4), and MRI showed normal cerebral vascular flow voids, suggesting lack of detectable vascular defect. *PCDH12* pathogenic variants have been recently linked to brain calcifications²¹ therefore we examined the available CT images. We found subtle brain

calcifications in six out of seven available CT studies (not shown), suggesting that calcifications can accompany DMJD due to *PCDH12* mutations.

PCDH12 is Involved in Neurite Outgrowth

PCDH12 is expressed ubiquitously in human tissues, including the nervous system (Fig. 4A), but expression was mainly documented in endothelial cells; therefore, we used induced pluripotent stem cells (iPSCs) to determine whether *PCDH12* is expressed in human neural cell lineages. Fibroblast cultures from two affected and one carrier individual (heterozygous father) from Family 1 were reprogrammed to iPSCs, verified upon expression of pluripotency markers and differentiation to three germ layers (not shown). *PCDH12* expression was undetectable in fibroblasts or iPSCs (not shown). Each iPSC line was differentiated into neural progenitor cells (NPCs) and endothelial cells separately. All NPC clones expressed early neural stem cell markers PAX6 and nestin. Similarly, all endothelial cell clones expressed the endothelial markers vWF, calponin and vinculin (Fig 4B). *PCDH12* showed expression in both neural and endothelial derivative cells. We obtained similar results from NPCs and endothelial cells derived from three different iPSC clones subjected to two independent differentiation rounds for each individual.

PCDH12 was expressed in differentiated cells from control (wild-type) and unaffected (heterozygous) individuals, both in NPCs and endothelial cells, but was undetectable in cells from affected individuals. This is in agreement with the analysis in cell culture (Fig 1C). Family 1 presented with c.2511delG, a pathogenic variant in the first exon leading to a predicted premature protein truncation, suggesting lack of *PCDH12* expression due to nonsense mediated decay. Moreover, all neural markers tested were exclusively found in NPCs, from both unaffected and affected individuals. Similarly, endothelial cells from both unaffected and affected individuals expressed endothelial but not neural markers (Fig 4B). We conclude that *PCDH12* is expressed in both endothelial and neural lineages, and lack of *PCDH12* does not notably impact lineage differentiation.

Recently it has been suggested that non-clustered protocadherins play roles in neurite outgrowth, therefore we investigated the possible neural phenotypes caused by lack of *PCDH12*. We utilized dissociated NPCs in culture to investigate the role of *PCDH12* in neurite outgrowth. We analyzed morphology of single NPCs plated at high-density by sparse GFP labeling (i.e. small percent of labeled cells). We found that mutant NPCs showed impaired neurite length (Fig. 4C), suggesting that neurite outgrowth is dependent on *PCDH12* expression.

Discussion

Protocadherins are implicated in the establishment and maintenance of neural circuits, and pathogenic variants in some protocadherin genes have been associated with neurodevelopmental and psychiatric disorders.^{12–18,29} Although recent advances have aided our understanding of the molecular and cellular mechanisms related to protocadherin regulation of functional neuronal circuitry, key questions remain unanswered.

Here we report fourteen cases in eight families with pathogenic variants in the non-clustered *PCDH12* in DMJD. Global developmental delay with severe cognitive impairment, hypotonia, dysmorphic facies, microcephaly, spasticity and seizures were among the common features. Magnetic resonance imaging studies obtained in affected individuals show a poorly defined diencephalic-mesencephalic junction with a characteristic malformation of the midbrain on axial sections. Additional imaging features include thinned corpus callosum and supratentorial calcifications.

Recently a founder allele in *PCDH12* was identified in Palestinian families with congenital microcephaly and intracranial calcifications, mimicking a congenital infection.^{20,21} The phenotype was reported similar to ‘Intracranial calcifications with congenital microcephaly’, which those authors grouped with recessive conditions due to pathogenic variants in a tight junction component, occludin (*OCLN*, MIM#251290)³⁰ or junctional adhesion molecule 3 (*JAM3*, MIM#613730).³¹ Here, we report that patients with *PCDH12* pathogenic variants not only present with calcifications and congenital microcephaly, but also abnormalities in white matter tracts. Consistent with these observations, we found *PCDH12* expression in both neural and endothelial cells. We performed brain MR-tractography studies on a family with *OCLN*-related microcephaly and found no evidence of white matter defects suggesting that the neural phenotype is not an obligate part of the brain calcification spectrum of conditions (not shown). Further studies are required to understand the precise role of *PCDH12* in brainstem development, or cross-talk between neurons and blood vessels during neural circuit formation.

In DMJD, MR-tractography studies evidenced the corticospinal tract originating within the cortex but then failed to project through the ventral forebrain to reach the level of the brainstem, suggesting that the ‘butterfly sign’ reflects a brainstem malformation with its origins in failed cortical projections. We identified additional patients with *PCDH12* pathogenic variants presenting with brainstem malformations, severe spasticity accompanied by paralysis, motor incoordination, and thinned corpus callosum, suggesting a generalized disruption of axonal tract formation. Development of mature axon tracts requires that axons navigate to find specific targets but also that axons have a continuous outgrowth in order to reach the target. A defect in either event could result in abnormal tracts. Here, we found defective neurite growth due to loss of *PCDH12* in NPCs derived from affected individuals. Therefore, we propose that individuals carrying *PCDH12* pathogenic variants display abnormalities in white matter tracts possibly due to defects in neurite outgrowth. This is in agreement with extensive literature showing that nonclustered protocadherins play roles in neurite outgrowth. *PCDH7*, *PCDH10*, *PCDH17*, *PCDH18*, and *PCDH20* mediate axon growth and extension.^{32–35} Moreover, *PCDH7*, *PCDH8* and *PCDH10* are implicated in dendritic spine and synapse density,³⁶ and *PCDH20* in conferring neuronal positioning.²⁹

Understanding the roles of the family of non-clustered protocadherins in brain development will require further study and the use of different models, including iPSCs. Our data adds *PCDH12* to the members of non-clustered protocadherins family involved in the establishment and maintenance of neural circuits during nervous system development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

This work was supported by the National Institutes of Health (NIH) R01NS048453 and R01NS098004 to J.G.G., P30NS047101 for imaging support, K99NS089943 to A.G.-G., the Yale Center for Mendelian Disorders U54HG006504, RC2NS070477 and the Gregory M. Kiez and Mehmet Kutman Foundation to M.G. Simons Foundation Grant 175303 and 275275, QNRF grant NPRP 6-1463-3-351 to J.G.G. and T.B.-O., and UMI HG008900 to the Broad Institute of MIT and Harvard Center for Mendelian Genomics (Broad CMG). We acknowledge the Yale Biomedical High-Performance Computing Center for data analysis and storage; the Yale Program on Neurogenetics and the Yale Center for Human Genetics and Genomics; the Center for Inherited Disease Research for genotyping; and the Simons Foundation Autism Research Initiative. Sequencing was provided in part by a gift from BGI and Illumina, Inc. to Rady Children's Hospital, San Diego for undiagnosed patients. Consortium for Autosomal Recessive Intellectual Disability (CARID) supported patient ascertainment.

Abbreviations:

DMJD	diencephalic mesencephalic junction dysplasia
PCDH	protocadherin
NPCs	neural progenitor cells
ECs	endothelial cells
iPSCs	induced pluripotent stem cells
WES	whole-exome sequence

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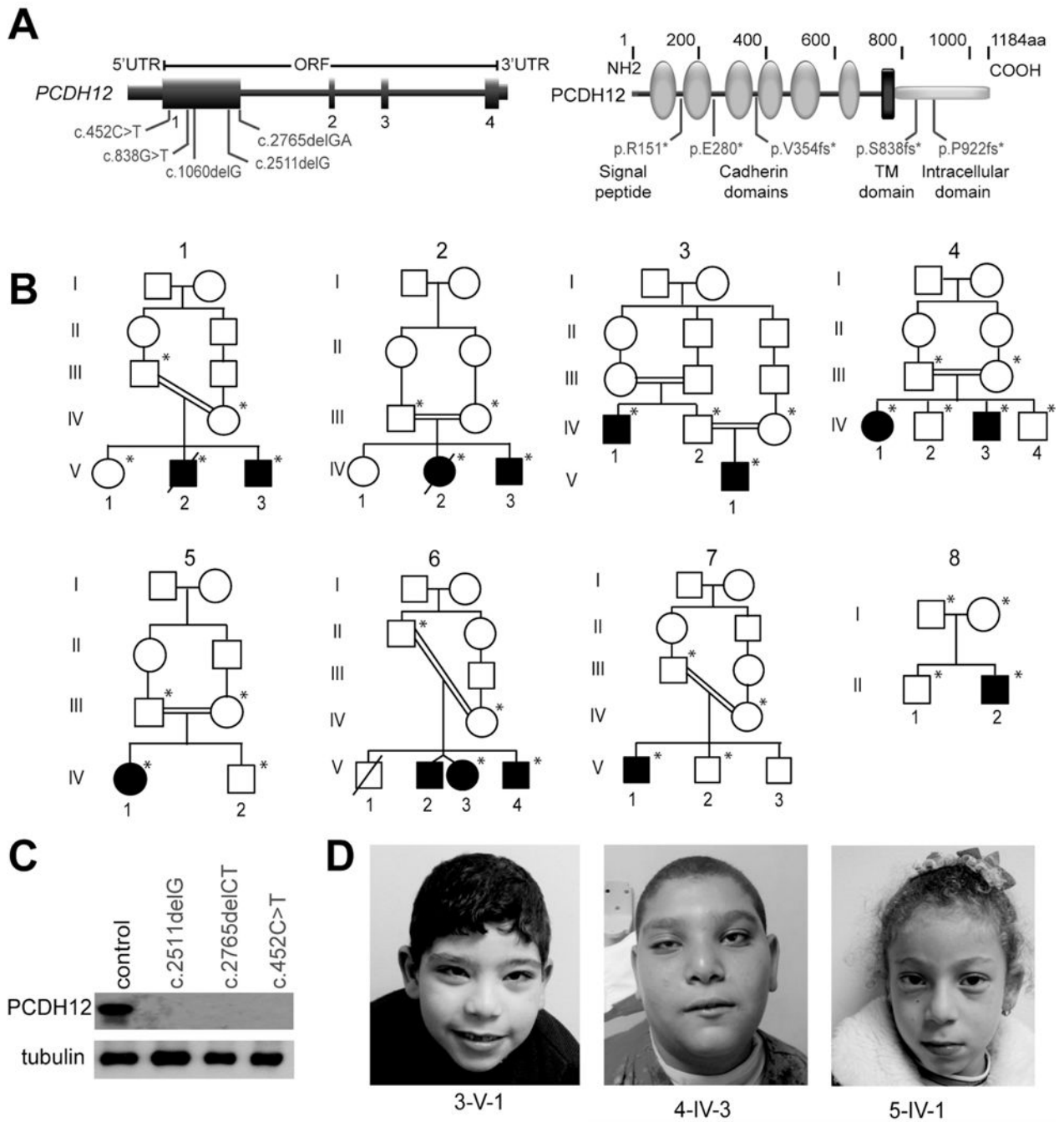


FIGURE 1. Bi-allelic pathogenic variants in *PCDH12* lead to microcephaly and spasticity. (A) Exons of *PCDH12* with location of the patient pathogenic variants (top). Location of pathogenic variants relative to predicted protein (bottom). Cadherin domains (circle), Transmembrane domain (TM). (B) Pedigrees of consanguineous Families 1, 2, 3, 4, 5, 6, 7, and non-consanguineous Family 8. (C) Impaired *PCDH12* expression in 293T cells transfected with vectors encoding pathogenic variants c.2511delG, c.2765delCT, and c.452C>T. (D) Pictures

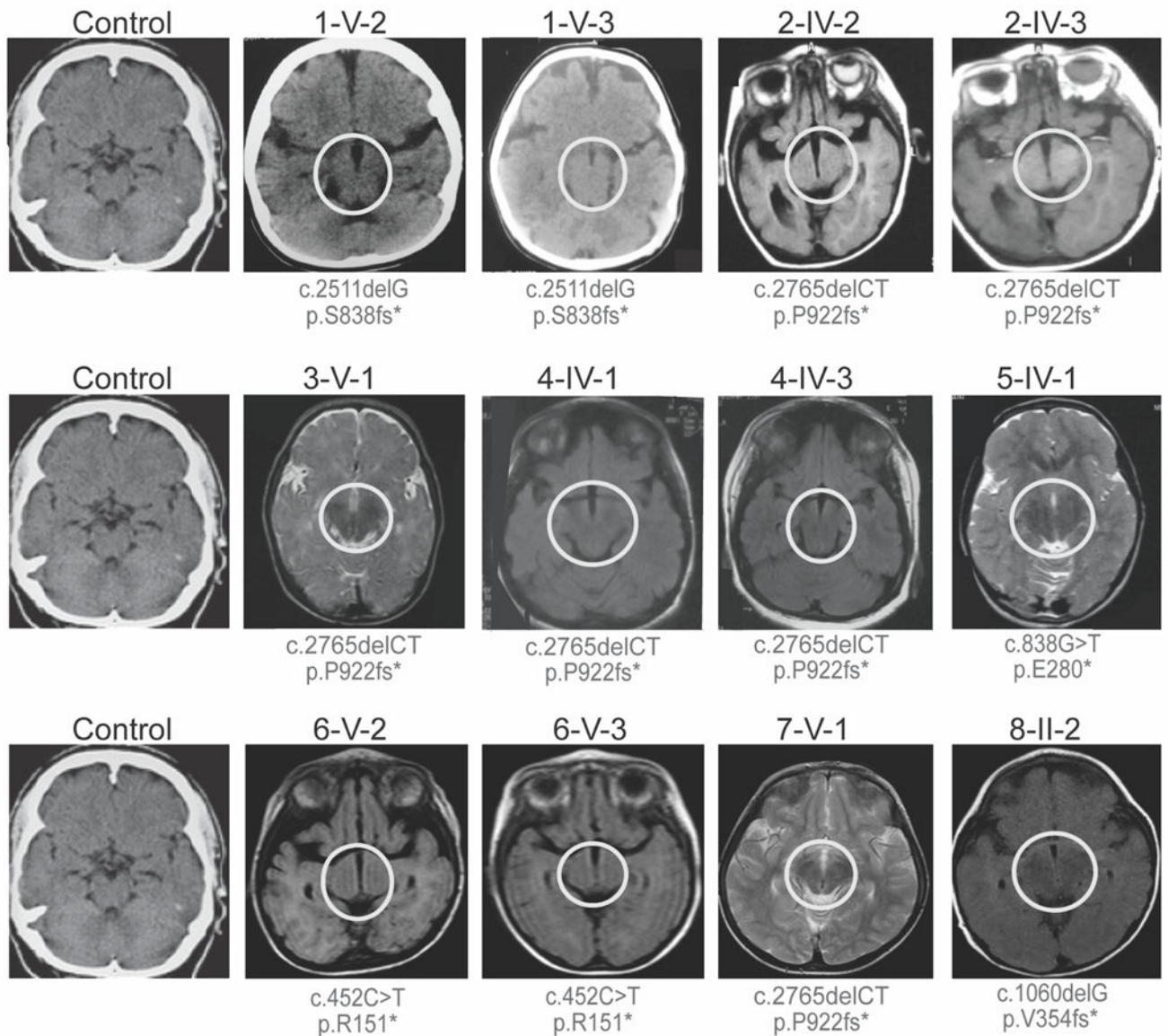
of affected members from Families 3, 4 and 5 showing the characteristic facial dysmorphism. Reproduced with permission of their parents.

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**FIGURE 2.**

Bi-allelic pathogenic variants in *PCDH12* lead to brainstem malformations. Head computed tomography (CT) or brain magnetic resonance imaging (MRI) of 12 affected individuals at the level of midbrain compared with control. All affected individuals showed brainstem malformations (circle). Butterfly sign was most obvious in the originally described families 1 and 2 (top row), consisting of an anterior midbrain cleft contiguous with the 3rd ventricle. In other families in which *PCDH12* pathogenic variants were identified from sequencing, the butterfly sign was not obvious (for example 5 and 8). *PCDH12* pathogenic variant in cDNA (c.) and protein (p.) corresponding to NM_016580 and NP_057664 Entrez IDs.

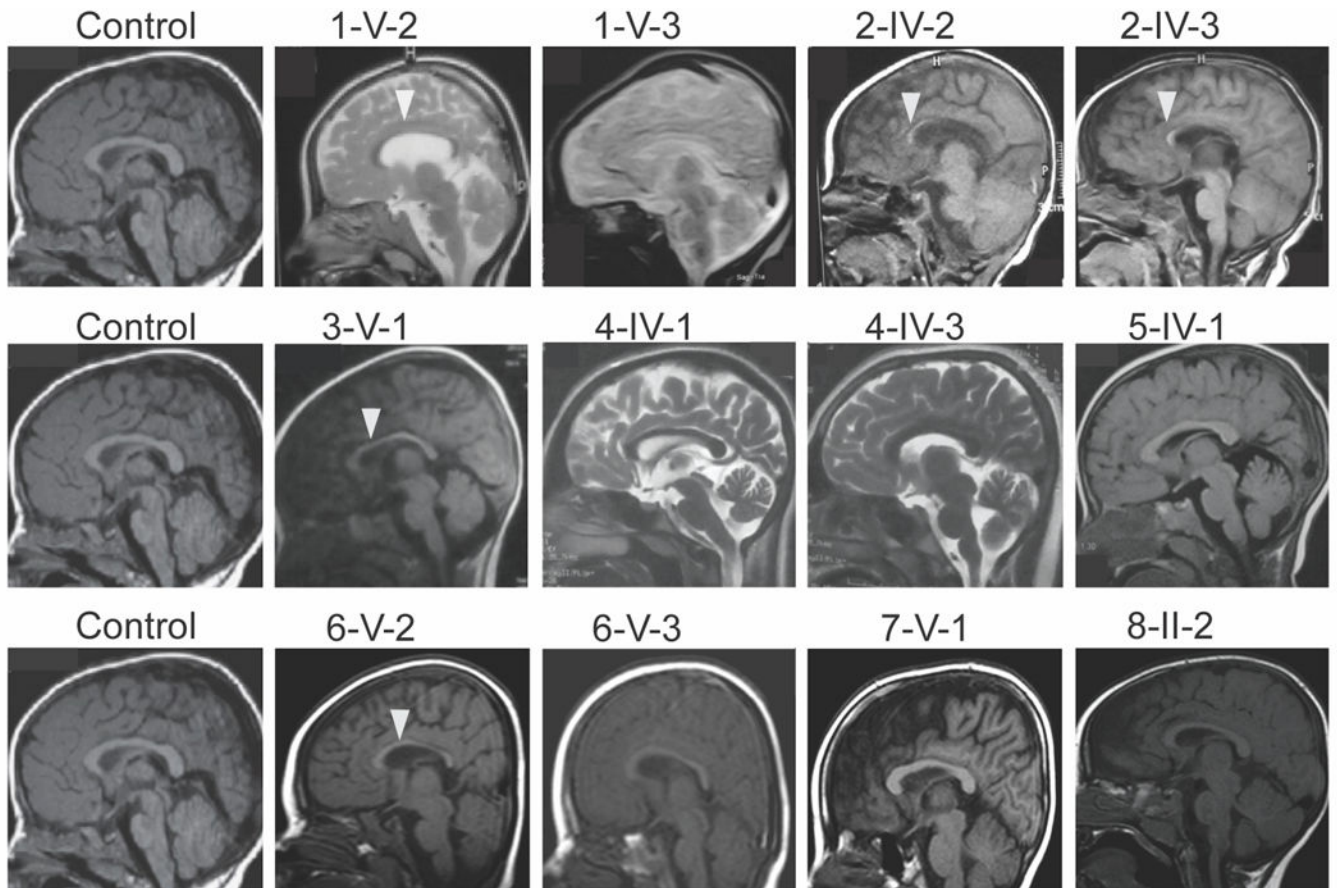


FIGURE 3. Bi-allelic pathogenic variants in *PCDH12* lead to defects in white matter tracts. Midline sagittal brain MRI showing thinned corpus callosum (arrowheads) in many of the affected individuals.

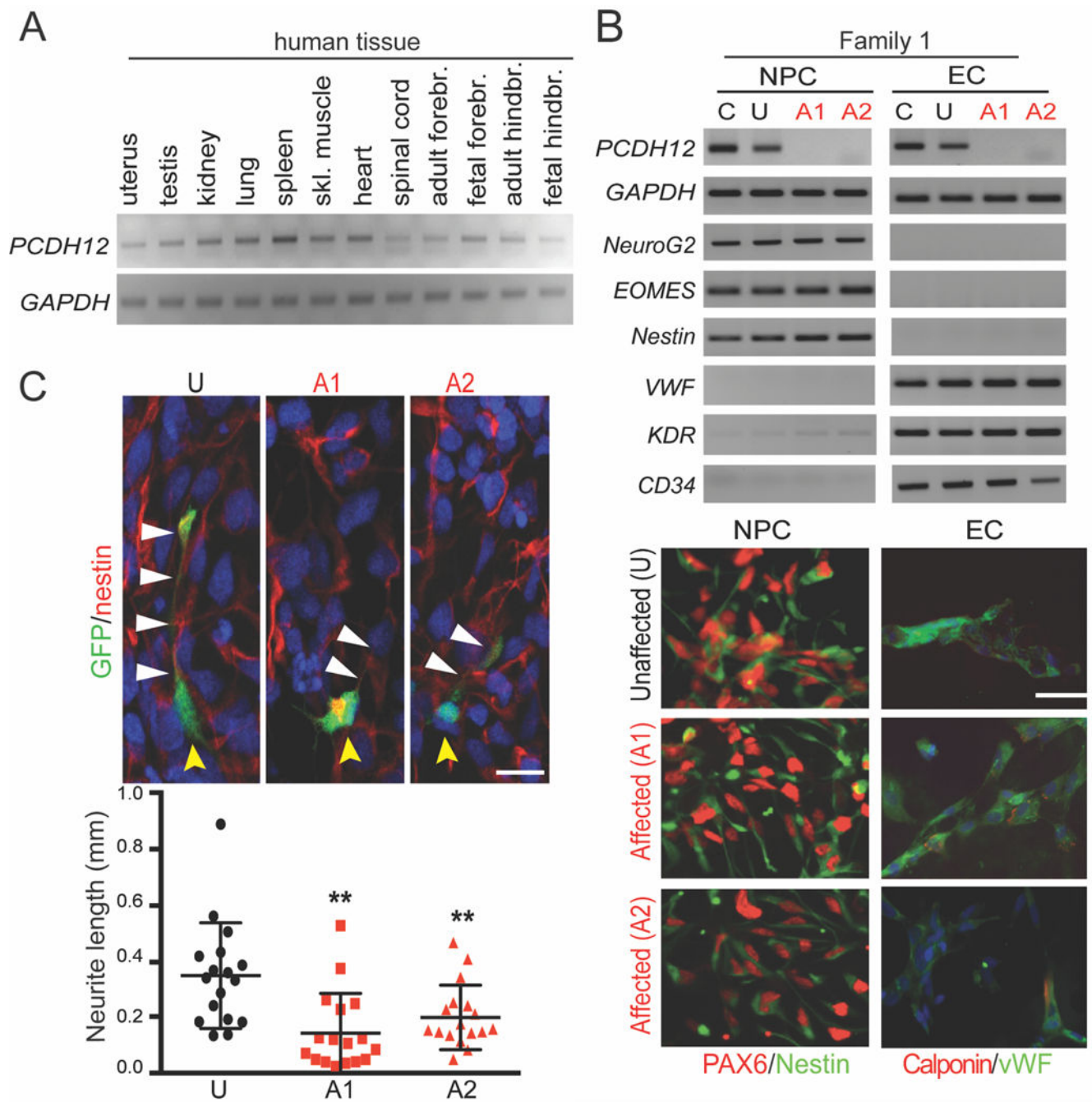


FIGURE 4. *PCDH12* is expressed in several cell populations during human brain development. (A) RT-PCR of *PCDH12* showing ubiquitous expression across human tissues and in fibroblasts. (B) Neural precursor cells (NPCs) and endothelial cells (ECs) derived from unaffected (U) and affected (A1 and A2) members of Family 1 are indistinguishable in differentiation. RT-PCR from NPCs and ECs showing *PCDH12* expression in control and unaffected but not in affected cells. Differential expression of cell-specific markers is not dependent on the genotype. Isolated NPCs are PAX6 and nestin co-positive. Isolated ECs are calponin and

vWF co-positive. No differences in immunostaining with neural and endothelial markers were observed. Scale bar, 400 μ m. (C) Single GFP-labeled NPCs showing reduced neurite length in cells from affected cells lacking PCDH12 (A1 and A2) compared with unaffected (U), at high cell density. Neurite length averaged 0.38 in unaffected and 0.15 or 0.21 μ m in affected (A1 and A2, respectively). Scale bar 10 μ m. Red: nestin. Green: GFP-labeled neurite, Yellow arrowhead: cell body. White arrowheads: neurite. Mean \pm SD. N 30 per genotype, 5 cells assessed in duplicates in 3 independent experiments. ** $p < 0.05$, one-way ANOVA.

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TABLE 1:Summarized Clinical Features of Patients with Pathogenic Variants in *PCDH12*

Clinical Finding	Patients, %
<i>Microcephaly</i>	100% (14 of 14)
<i>Intellectual disability</i>	100% (14 of 14)
<i>Facial dysmorphism</i>	92% (13 of 14)
<i>Seizures</i>	64% (9 of 14)
<i>Recurrent unexplained fever</i>	64% (9 of 14)
<i>Vasomotor instability</i>	57% (8 of 14)
<i>Ataxia</i>	42% (6 of 14)
<i>Autistic features</i>	
<i>Pyramidal tract signs</i>	28% (4 of 14)
<i>Hyperreflexia</i>	64% (9 of 14)
<i>Spasticity</i>	28% (4 of 14)
<i>Hypotonia</i>	28% (4 of 14)
<i>Cranial nerve findings</i>	
<i>Strabismus</i>	28% (4 of 14)
<i>Dysphagia</i>	28% (4 of 14)
<i>Minimal to absent tracking</i>	21% (3 of 14)
<i>Squinting</i>	14% (2 of 14)
<i>Brain imaging findings</i>	
<i>DMJD</i>	100% (14 of 14)
<i>Corpus callosum hypoplasia</i>	92% (13 of 14)
<i>Brain calcification</i>	85% (6 of 7)
<i>Ventriculomegaly</i>	57% (8 of 14)