Brain calcifications and *PCDH12* variants

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Supplemental data at Neurology.org/ng

ABSTRACT

Objective: To assess the potential connection between PCDH12 and brain calcifications in a patient carrying a homozygous nonsense variant in *PCDH12* and in adult patients with brain calcifications.

Methods: We performed a CT scan in 1 child with a homozygous *PCDH12* nonsense variant. We screened DNA samples from 53 patients with primary familial brain calcification (PFBC) and 26 patients with brain calcification of unknown cause (BCUC).

Results: We identified brain calcifications in subcortical and perithalamic regions in the patient with a homozygous *PCDH12* nonsense variant. The calcification pattern was different from what has been observed in PFBC and more similar to what is described in in utero infections. In patients with PFBC or BCUC, we found no protein-truncating variant and 3 rare (minor allele frequency <0.001) *PCDH12* predicted damaging missense heterozygous variants in 3 unrelated patients, albeit with no segregation data available.

Conclusions: Brain calcifications should be added to the phenotypic spectrum associated with *PCDH12* biallelic loss of function, in the context of severe cerebral developmental abnormalities. A putative role for *PCDH12* variants remains to be determined in PFBC. ***Neurol Genet* 2017;3: e166; doi: 10.1212/NXG.0000000000000166**

GLOSSARY

BCUC = brain calcification of unknown cause; **ExAC** = Exome Aggregation Consortium; **PFBC** = primary familial brain calcification.

A homozygous nonsense *PCDH12* variant has recently been reported in consanguineous families, where the affected children had congenital microcephaly, epilepsy, and profound global developmental disability.[1] Fetal MRI and USG showed dysplastic elongated masses in the midbrain-hypothalamus-optic tract area and hyperechogenic perithalamic foci. *PCDH12* encodes a protocadherin associated with membrane physical stability, adhesion, and vasculature maintenance and has recently been pointed out as a candidate gene for primary familial brain calcification (PFBC). PFBC is characterized by the presence of calcifications affecting primarily the basal ganglia, in the absence of secondary cause.[2] Clinical manifestations include movement disorders, cognitive impairment, psychiatric disturbances, and headache, most frequently beginning during adulthood.[2,3] Heterozygous variants causing autosomal dominant PFBC in up to 50% of the families were identified in 4 genes: *SLC20A2*, *PDGFRB*, *PDGFB*, and *XPR1*.[4–8] We previously searched for genes with a cerebral expression pattern similar to the PFBC major

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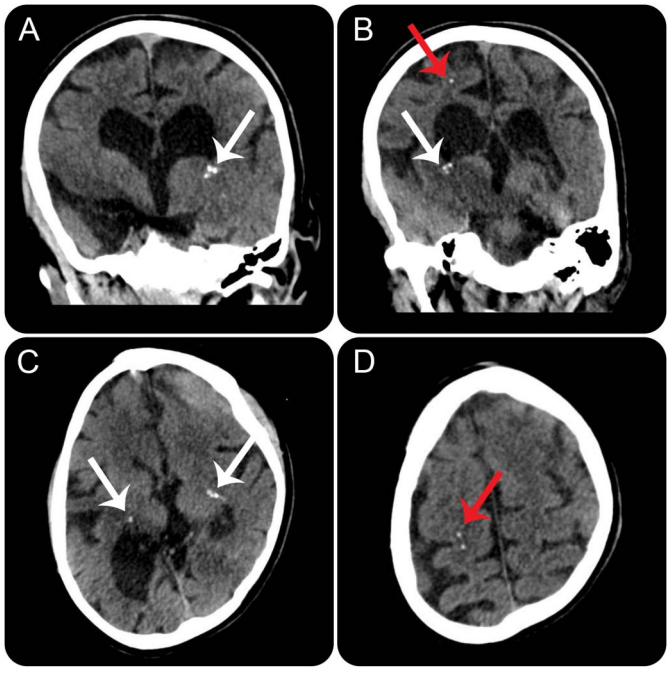
causative gene *SLC20A2* using the Allen Brain Atlas (brain-map.org/),[9,10] observing a higher *SLC20A2* expression in regions affected by calcifications in PFBC. *PCDH12* was singled out with the highest significant correlation,[10] and a follow-up analysis with additional brains still shows *PCDH12* as the most similar pattern to *SLC20A2*, even when compared with the other known PFBC causative genes (table e-1 at Neurology.org/ng).

To evaluate the potential link between *PCDH12* and brain calcifications, (1) we performed a CT scan in a patient reported to carry a homozygous nonsense *PCDH12* variant and (2) we screened DNA samples from patients with PFBC or brain calcifications of unknown cause (BCUC).

METHODS

CT imaging in *PCDH12* homozygous variant carriers. In the original report, patients with symmetric intrauterine growth retardation, severe microcephaly, visual impairment, dystonia, epilepsy, and profound developmental disability were shown to carry a *PCDH12* c.995T>A, p.R839X homozygous variant.[1] This variant is considered to be pathogenic when carried at the homozygous state following the American College of Medical Genetics and Genomics and the Association for Molecular Pathology recommendations.[11] Brain imaging revealed midbrain hypothalamus dysplasia and significant periventricular and/or periventricular hyperechogenicity. Fetal USG and MRI did not enable to determine whether these foci are eventually calcifications. Therefore, we performed a brain CT scan in individual III-1, family B from the original pedigree.[1]

Figure Brain CT imaging of a patient carrying the PCDH12 c.995T>A, p.R839X homozygous variant



(A, B) Coronal sections. (C, D) Transversal sections. Spot calcifications affecting perithalamic regions (white arrows, A–C) and subcortical regions (red arrows, B, D).

***PCDH12* screening in patients with brain calcification.** We included a total of 79 worldwide adult cases with brain calcifications that were referred to 5 centers of expertise, negatively screened for the known PFBC causative genes (supplemental data). Of these, 53 cases matched the clinical inclusion criteria for PFBC (detailed previously in reference 3). Briefly, these cases exhibited at least bilateral basal ganglia calcifications and no secondary cause. The remaining 26 patients were included on a neuropathologic basis if they presented moderate-to-severe basal ganglia calcifications. Note that calcifications also involved other brain regions in almost all cases and that other causes of brain calcifications could not be excluded in these patients, thereafter referred as having BCUC. All patients were screened for pathogenic variants by sequencing all coding exons of *PCDH12* (reference transcript: NM_016085.3). Bioinformatics predictions were performed using direct access to Polyphen2 HumDiv,[12] SIFT,[13] and Mutation Taster[14] tools, and the minor allele frequency (MAF) was checked at the Exome Aggregation Consortium (ExAC) website accessed in August 2016 (exac.broadinstitute.org/).[15] Detailed inclusion criteria and sequencing methods are provided in supplemental data.

Standard protocol approvals, registrations, and patient consents. All patients provided written informed consent for genetic analyses.

RESULTS

CT of a *PCDH12* homozygous variant carrier. CT is the reference imaging to identify brain calcification, so we used it to determine the nature of the hyperechogenic foci identified in a patient with a homozygous nonsense p.R839X *PCDH12* variant.[1] We identified spots of perithalamic calcification located in the posterior arms of the internal capsules and in juxtacortical right white matter (figure).

***PCDH12* screening in patients with brain calcification.** As we provided evidence that *PCDH12* biallelic loss of function is associated with brain calcification and given the high level of coexpression with the PFBC major causative gene *SLC20A2*, we next screened this gene in a group of patients with PFBC or BCUC. Among the 79 patients with PFBC or BCUC, we did not identify any protein-truncating variant (nonsense, splice site, or frameshift insertion/deletion). However, we detected 4 rare (MAF <0.001 in ExAC) heterozygous *PCDH12* missense variants in 4 unrelated patients: c.163C>G, p.(R55G); c.440G>T, p.(S147I); c.995T>A, p.(I332N); and c.3271G>A, p.(G1091S) (table 1). Three were predicted damaging by at least 1 in silico tool, while variant p.R55G was predicted benign by all 3 tools.

The c.440G>T, p.(S147I) variant had an MAF of 2.5e-05 in the ExAC database and was exclusively found in 3 individuals with the same ancestry as the patient (classified in ExAC as European non-Finnish). Two of the 3 in silico tools (Mutation Taster and Polyphen2 HumDiv, but not SIFT) predicted a damaging effect for this change to the protein

Table 1 Rare *PCDH12* variants identified in a series of 79 patients with PFBC or BCUC

Location (Ghrc37)	cDNA change[a]	Protein change[a]	ExAC frequency[b]	SIFT prediction	Polyphen2 HumDiv prediction	Mutation Taster prediction	PhyloP
chr5:141337254	c.163C>G	p.(R55G)	6.1e-04	Tolerated	Benign	Polymorphism	−1.01
chr5:141336977	c.440G>T	p.(S147I)	2.5e-05	Tolerated	Possibly damaging[c]	Disease causing[c]	2.14[c]
chr5:141336422	c.995T>A	p.(I332N)	1e-04	Deleterious[c]	Probably damaging[c]	Disease causing[c]	4.48[c]
chr5:141325230	c.3271G>A	p.(G1091S)	3.3e-05	Deleterious[c]	Probably damaging[c]	Disease causing[c]	4.81[c]

Abbreviations: BCUC = brain calcification of unknown cause; cDNA = complementary DNA; ExAC = Exome Aggregation Consortium; PFBC = primary familial brain calcification.
[a] Accession number: NM_016085.3.
[b] ExAC minor allele frequency assessed in August 2016.[15]
[c] Values are above each threshold.

function. DNA from relatives was not available for segregation analysis. This variant is located in the second cadherin tandem repeat domain (EC2) (NCBI accession cd11304) and, therefore, could affect homophilic adhesive behavior and calcium-dependent cell adhesion.[16]

The c.995T>A, p.(I332N) and c.3271G>A, p.(G1091S) variants are both predicted damaging by all 3 in silico tools. The p.I332N variant was reported with an overall MAF of 0.0001 in ExAC, found in 12 individuals of East Asian ancestry (the patient was born in Southeastern Asia) and 1 individual of European non-Finnish ancestry. The p.G1091S variant has an overall MAF of 3.3e-05, found in 1 individual of European non-Finnish ancestry (same as the patient) and 3 individuals of South Asian ancestry. DNA from relatives was not available for segregation analysis of any variant. Variant p.I332N is also located in a cadherin tandem repeat domain, namely EC3. However, p.G1091S variant is located in a highly conserved site in the cytoplasmic domain, which has a unique sequence among the cadherin family. Unlike the other cadherins, the cytoplasmic domain of PCDH12 does not interact with catenins, and it is involved in cellular processes other than cell junction, such as regulation of gene expression and signaling pathways.[17] Clinical details of all 3 predicted damaging variant carriers are provided in the supplemental data.

DISCUSSION We show here that a homozygous nonsense *PCDH12* variant, detected in patients with severe developmental delay and microcephaly,[1] is associated with brain calcifications. This feature should therefore be added to the phenotypic spectrum of this rare disorder. The pattern of calcifications is, however, different from the typical findings in PFBC, where calcifications always affect at least both pallidum,[3] and resembled to those observed in various neuroinfectious prenatal conditions, such as TORCH infections.[18] Brain calcification is a highly informative feature on brain imaging of children with neurodevelopmental disorders.[18] Although CT is the reference imaging tool for detecting and assessing calcifications, MRI is the primary imaging tool for the detection of all other brain abnormalities in the absence of radiation. T2* or susceptibility-weighted images increase the diagnostic performance of MRI for calcification compared with the other sequences. However, they can sometimes miss small calcifications, and they are still complementary with CT to describe precise shape and intensity and to definitely conclude on the differential identification with iron deposits.[19,20] In our patient, neither T2* nor susceptibility-weighted images were available.

In the original report, the efficiency of nonsense-mediated decay has been measured as 84%, suggesting a strong loss of function. The patients carrying the nonsense *PCDH12* variant in a homozygous state may still express little amount of the truncated protein, but no full-length PCDH12. This supports the hypothesis that loss of function of PCDH12 is the mechanism leading to the patient's phenotype, including brain calcification.

In a candidate gene approach, we searched for rare *PCDH12* variants in PFBC and BCUC patients and found no protein-truncating variants. Three heterozygous missense variants, predicted damaging by at least one of the tools, were identified in 2 patients with PFBC and 1 patient with BCUC. Given the fact that biallelic loss of *PCDH12* function leads to a severe neurodevelopmental phenotype, it is unlikely that these variants have a dominant-negative effect. However, as they are missense variants, their putative effect on protein function is hard to predict, and it remains possible that they are responsible for loss of function, gain of function, or have a neutral effect on protein function. The frequencies of theses variants in the patients' respective populations as estimated in ExAC are not inconsistent with a causative effect, as they are in the same frequency ranges as other disease-causing variants in *SLC20A2*.[8] Because neither segregation nor functional data are available, it is not possible to conclude about their pathogenicity at this stage.

Besides PFBC, brain calcifications can be detected in other numerous distinct conditions, such as

systemic phosphocalcic metabolism disorders of inherited or acquired cause, in utero or postnatal infections, interferonopathies, inborn errors of metabolism, and other rare inherited diseases.[21] Calcifications are believed to be related to increased type-I interferon response in both in utero viral infections and interferonopathies.[22] Several of these clinical presentations, including TORCH in utero infections and typical Aicardi-Goutières syndrome, are similar to the ones observed in the *PCDH12* homozygous carriers. In other conditions, mutations in *OCLN* and *JAM3* genes, encoding endothelial cell adhesion proteins, result in microangiopathy associated with calcifications.[18,23,24] Given the known function of PCDH12, we postulate that similar mechanisms could be associated with the calcifications observed in the *PCDH12* homozygous loss-of-function carriers.

PCDH12 is a protocadherin associated with membrane physical stability and adhesion.[25] A *Pcdh12* knockout mouse model revealed several age-independent vessel impairments, such as ramifications of medial elastic lamellae and increased inner diameter and circumferential mid-wall stress.[26] PCDH12 has been widely studied as a key-player cadherin involved in placental maintenance and also a preeclampsia biomarker; however, little is known about its involvement in brain physiology. It is conceivable that mutations in PCDH12 and SLC20A2, which share similar expression patterns in the brain, might lead to similar phenotypes. Of interest, *Slc20a2* knockout mice developed not only brain calcifications but also fetal growth restriction, lower birth viability, and placental calcification associated with thickened basement membranes.[27] In both mouse models, the placental phenotype and the vascular impairment are additional putative links between SLC20A2 and PCDH12, which deserve additional studies on mouse models.

PCDH12 biallelic loss of function causes a severe neurodevelopmental phenotype associated with brain calcifications. Rare predicted damaging heterozygous *PCDH12* variants were identified in patients with PFBC or BCUC here, but whether they are associated with brain calcification or not remains to be determined. To address this question, follow-up studies will be necessary including screening other series, assessing the segregation of rare variants and functional consequences.

AUTHOR CONTRIBUTIONS

Collection and interpretation of data: Gaël Nicolas, Monica Sanchez-Contreras, Eliana Marisa Ramos, Roberta R. Lemos, Joana Ferreira, Denis Moura, Maria J. Sobrido, Anne-Claire Richard, Alma Rosa Lopez, Andrea Legati, Jean-François Deleuze, Anne Boland, Olivier Quenez, Pierre Krystkowiak, Pascal Favrole, Daniel H. Geschwind, Adi Aran, Reeval Segel, Ephrat Levy-Lahad, Dennis W. Dickson, Giovanni Coppola, Rosa Rademakers, and João R.M. de Oliveira. Manuscript draft: Gaël Nicolas, Monica Sanchez-Contreras, Eliana Marisa Ramos, Roberta R. Lemos, and João R.M. de Oliveira. Critical revision: Gaël Nicolas, Monica Sanchez-Contreras, Eliana Marisa Ramos, Roberta R. Lemos, João R.M. de Oliveira, and Giovanni Coppola. Study design and supervision: João R.M. de Oliveira, Gaël Nicolas, Giovanni Coppola, and Rosa Rademakers.

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